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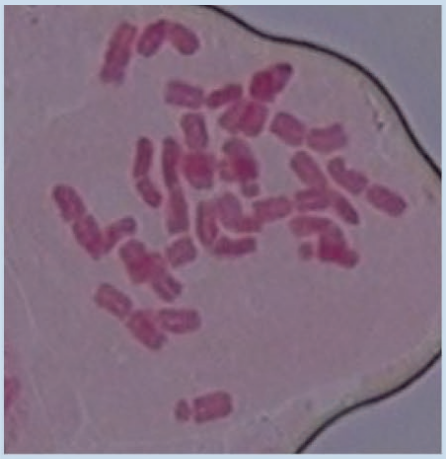

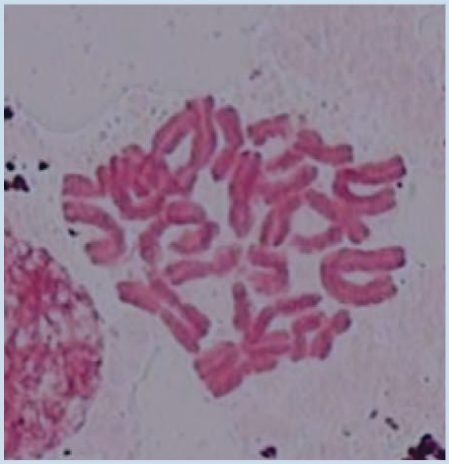

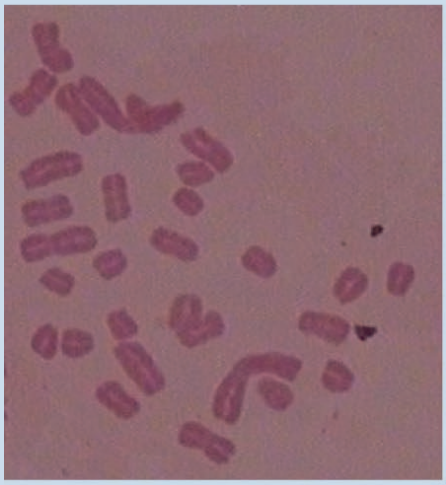

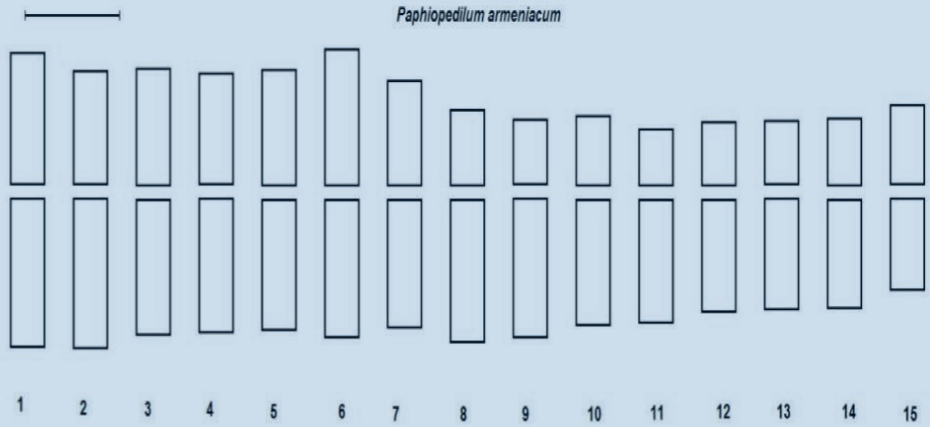

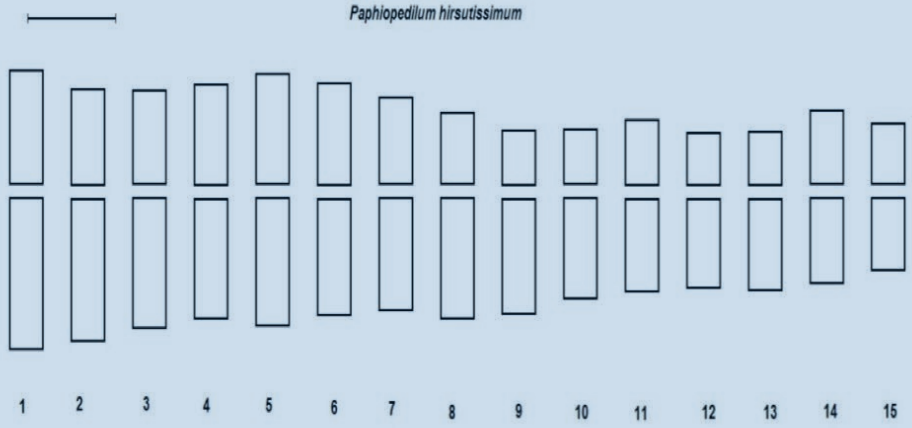

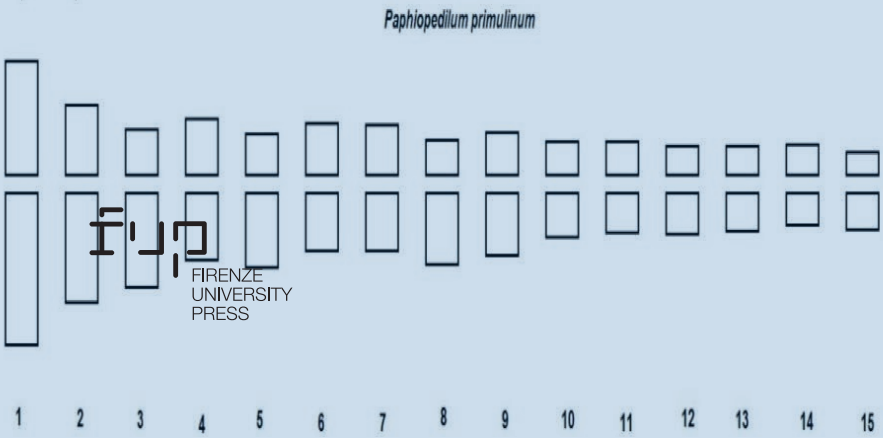

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Caryologia. International Journal of Cytology, Cytosystematics and Cytogenetics
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Cover: figure from the article inside by Titien Ngatinem Praptosuwiryo, R. Vitri Garvita, Elizabeth Handini, Izu Andry Fijridiyanto, Joko Ridho Witono. Karyological studies of four species of Lady's Slipper Orchids (*Paphiopedilum*) collected in the Bogor Botanical Garden, Indonesia.

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Meiotic behavior during microsporogenesis, responsible for male sterility in some species of *Salvia* sect. *Aethiopis* in Iran

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Abstract. The genus *Salvia* L. (Lamiaceae: tribe Mentheae) contains about 1000 species. Section *Aethiopis* in this genus has a distribution in the Mediterranean and central Asia and contains about 34 species in Iran. This study aimed to investigate the chromosome number and meiotic behavior in five species of *Salvia* sect. *Aethiopis*. To this end, pollen mother cells were used, and the squash method was employed. The results showed that *S. persepolitana* and *S. spinosa* had a chromosome number of 2n=2x=20, while *S. sclarea*, *S. hypoleuca*, and *S. limbata* had a chromosome number of 2n=2x=22. The study of meiotic behavior revealed the presence of abnormalities such as chromosome stickiness, cytomixis, non-synchronous segregation, chromosome bridges, laggard chromosomes, formation of micronuclei in tetrad cells, formation of tripolar cells, and pentapolar with different frequencies in the studied species. The meiotic index was reported as the highest in *S. persepolitana* and the lowest in *S. hypoleuca*. Pollen fertility was also affected by meiotic abnormalities, showing a decreasing trend from *S. persepolitana* to *S. spinose*. Meiotic abnormalities may have played a role in the evolution of aneuploidy and polyploidy in the *Salvia* genus.

Keywords: meiotic behavior, meiotic index, pollen mother cells, pollen fertility, *Salvia* sect. *Aethiopis*.

INTRODUCTION

The genus *Salvia* L. from the Lamiaceae, with about 900-1000 species, is spread throughout the old and new world, in subtropical and temperate regions. Western Asia and Mediterranean areas are considered the main distribution center of this genus (Sheidai et al. 2010; Celep et al. 2020). This genus is considered a suitable model for biological diversity and adaptive radiation studies (Standley and Williams, 1973; Wu and Li 1982). Salvia genus has about 55 species in Iran and they are distributed in tropical to arctic areas (Hedge 1982a).

Bahattacharya (1978) suggests that the *Salvia* species are recent and advanced members of a complex group, due to their highly irregular cytological behavior, advanced karyotype, and cytological numerical instability. Due to the small size of chromosomes in *Salvia*, chromosomal studies in this

genus are limited. Most of the studies carried out in *Salvia*, investigated the mitotic chromosomes and karyotype, and there is little information about the behavior of the chromosomes of this genus during meiosis and its chromosomal abnormalities. The behavior of meiotic chromosomes in different plant genera has been studied by scientists. Chromosome stickiness aberration is one of the common abnormalities in pollen mother cells. The visual appearance of stickiness varies from mild when only a few chromosomes are involved, to severe when it involves all the genome, especially during the formation of pachytene nuclei, and may even lead to chromatin destruction (Pagliarini 1990). Lagging chromosomes may be the result of delayed completion of karyokinesis (Pagliarini 1990). If lagging chromosomes do not reach the poles in time, they may cause the formation of micronuclei, micro-pollen, and pollen grains with unequal chromosome numbers. Such gametes may lead to aneuploidy (Utsunomiya et al. 2002; Defani-Scoarize et al. 1995). Chromosomes that form micronuclei during meiosis are separated by reaching the microspore wall and forming a bud called microspores. The separated microspores form small and non-viable pollen grains (Baptists-Giacomoelli et al. 2000). Meiotic aberrations lead to abnormal microsporogenesis like dyads, triads, tetrads with or without micronuclei (Tantray et al. 2021). Abnormalities such as triad and pentad may be due to cytomixis (Soodan and Wafai 1987). There are various other abnormalities in different stages of meiosis, which have been discussed further in *Salvia* species. In the chromosomal study of 19 species of the *Salvia* genus, Epling et al. (1962) examined the meiosis of natural hybrids only between *Salvia apiana* and *S. mellifera*. Systematic and evolutionary aspects of the genus in the light of the cytogenetic data were carried out in 13 species of *Salvia* from Argentina by Alberto et al. (2003). Sheidai et al. (2010), studied the behavior of the chromosomes in the meiosis of ten species of *Salvia*. Ranjbar et al. (2015), studied chromosome numbers and meiotic behavior in 12 species of *Salvia* from Iran. Alijanpoor and Safaeishakib (2023), studied cytomixis and other meiosis abnormalities in three species of *Salvia* (*S. nemorosa*, *S. staminea*, *S. verticellata*). The studies reported the number of chromosomes of different species of this genus as 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 36, 38, 42, 44, 46, 52, 54, 56, 60, 64 (Epling et al. 1962; Vij and Kashyap 1976; Haque 1981; Sheidai et al., 2010; Eroglu et al. 2021; Martin et al. 2022). The chromosome base numbers observed in *S.* sect. *Aethiopis* include x = 8, 9, 10, and 11. The majority of species displayed a chromosome count of 2n = 22, with a lower percentage exhibiting 2n = 20. The base numbers x = 8 (in *S. moorcroftiana*) and x = 9 (in *S. verbascifolia*) were less frequent. Polyploidy has been reported in *S. ceratophylla* and *S. desoleana*, with a chromosome count of 2n = 4x = 44. (Afzal-Rafii 1980; Diana Corrias 1983; Ranjbar et al. 2015).

In the present study, the variable chromosome numbers in 5 species of *S.* sect. *Aethiopis* from different regions of Qazvin province, Iran were recorded. The processes of male meiosis and various abnormalities in microsporogenesis were investigated in detail for all studied species. Furthermore, the relationships of the irregular meiotic divisions with the meiotic Index, and male sterility are discussed.

MATERIAL AND METHODS

Plant material

During 2021 to 2022, plant materials were collected in various localities around Qazvin province, with fresh flower buds being the primary focus. Table 1 provides details of the locations where the 5 *Salvia* species (including *S. persepolitana* Boiss., *S. spinosa* L., *S.*

Table 1. Taxa studied of *Salvia* sect. *Aethiopis* species in Iran, Qazvin province.

Species	section	Locality	Collector	2n	x
S. persepolitana	*Aethiopis*	Iran, Qazvin: Abegarm to Avaj, 35°36'N, 49°12'E	Hajmoradi	20	10
S. spinosa	*Aethiopis*	Iran, Qazvin province, Tarom-e-Sofla 36°34'N, 49°31'E	Hajmoradi	20	10
S. sclarea	*Aethiopis*	Iran, Qazvin province, near Moallem Kalayeh 36°26'N, 50°28'E	Hajmoradi	22	11
S. hypoleuca	*Aethiopis*	Iran, Qazvin province, Chizeh village 36°34'N, 49°01'E	Hajmoradi	22	11
S. limbata	*Aethiopis*	Iran, Qazvin province, Alamut-e-Sharghi 36°24'N, 50°14'E	Hajmoradi	22	11

sclarea L., *S. hypoleuca* Benth. and *S. limbata*) used in this study were collected. Herbarium specimens were identified using the Oriental Flora (Boissier 1879), Flora Iranica (Hedge 1982a), and Turkish Flora (Hedge 1982b).

Cytological study

To conduct the cytogenetic study, a total of 15 flower buds were collected from a minimum of five plants at an appropriate stage of development. These buds were then fixed in a solution consisting of 96% ethanol, chloroform, and propionic acid (in a ratio of 6:3:2) for 24 hours at room temperature. After fixation, the buds were stored in 70% alcohol at 4°C until they were ready to be used. For studying pollen mother cells (PMCs), the anthers from the buds were squashed and stained with a 2% acetocarmine solution. To make the slides permanent, Venetian turpentine was applied. Chromosome photographs were taken using an Olympus BX-41 photomicroscope at an initial magnification of 1000. Chromosome counts were determined by examining well-spread metaphases in intact cells through direct observation and also by analyzing the photomicrographs.

Meiotic Index (MI)

The meiotic index (MI) was calculated by dividing the number of normal tetrads by the total number of observed tetrads and multiplying by 100 (Tedesco et al. 2002).

Pollen fertility

To estimate pollen fertility, the ability of pollen to stain was assessed. Pollen grains were obtained from the flowers of studied herbarium specimens and then stained with a mixture of acetocarmin and glycerin (1:1). After being stored at room temperature for 24-48 hours, 1000 pollen grains per flower were sampled to determine stainability. Documentation and examination of the slides were performed using an Olympus BX-51 photomicroscope. Pollen grains were considered fertile if they were well-stained and infertile if they were empty or unstained. The percentage of pollen fertility was calculated by dividing the total number of fertile pollen grains by the total number of pollen grains and multiplying the result by 100.

RESULT

Meiotic abnormalities

In studied species of *Salvia* sect. *Aethiopis*, chromosome numbers and meiotic behavior were determined and their cytological features are summarized in Table 2. Figure 1 provides illustrations of the chromosomes and their abnormalities. The species studied were diploid and had 2n = 20 and 22 chromosome numbers. PMCs at diakinesis without any abnormality showed the normal chromosome pairing as 10 bivalents in *S. persepolitana* and *S. spinosa* (2n = 20) and 11 bivalents in *S. sclarea, S. hypoleuca, and S. limbata* (2n = 22).

A total of 589 cells in diakinesis and metaphase I, 1029 cells in anaphase I and telophase I, 451 cells in metaphase II, and 1619 cells in anaphase II and telophase II were analyzed in the studied species of *Salvia*. The meiotic irregularities observed in the studied populations included: chromosome stickiness, cytomixis, non-synchronous segregation, chromosome bridges, laggard chromosomes, formation of micronuclei in tetrad cells, formation of tripolar cells, and pentapolar which have been discussed below (Figure 1).

Chromosome stickiness is one of the chromosomal abnormalities that is seen more frequently than other abnormalities in all studied species during diakinesis/metaphase I and metaphase II. *S. hypoleuca* showed the highest percentage of this abnormality at 21.3±0.12, while *S. sclarea* showed the lowest at 10.7±0.07 during diakinesis/metaphase I. In metaphase II, the highest percentage of stickiness was observed in *S. spinosa* and the lowest in *S. limbata* (4.8±0.03 and 1.3±0.02, respectively). The physical appearance of stickiness varies from mild, when only a few chromosomes are involved, to severe, when it may involve the entire genome, and may even lead to the destruction of chromatin. The transfer of chromosomes or chromatin content from one cell to one or more other cells is called cytomixis, which occurs through cytoplasmic bridges. This abnormality was observed in three out of the five studied species, with *S. sclarea* showing the highest frequency and *S. hypoleuca* showing the lowest. In *S. sclarea*, the transfer of chromosomes through this phenomenon was observed partially or completely, involving the entire genome. Cytomixis abnormalities had a higher frequency in meiosis I than in meiosis II (Figure 1C). The phenomenon of cytomixis was not reported in any stage of meiosis in *S. persepolitana* and *S. limbata.*

Chromatin bridges with various thicknesses are another anomaly observed in most of the studied species during anaphase I and II stages (Figure 1D), while *S. sclarea* did not show this abnormality in any of the

Table 2. The number of pollen mother cells (PMCs) analyzed and the percentage of PMCs meiotic behavior in *Salvia* sect. *Aethiopis*.

Meiotic characters/taxa	*S. persepolitana*	*S. spinosa*	*S. sclarea*	*S. hypoleuca*	*S. limbata*
Total cell number	704	751	746	758	729
D/MI	110	123	103	136	117
% D/MI	15.6	17.2	13.8	17.9	16
% Sticky chromosome	14.2±0.03	18.4±0.14	10.7±0.07	21.3±0.12	15.8±0.03
% Cytomixis	-	3.4±0.04	4.7±0.12	-	-
AI/TI	184	209	214	195	227
% AI/TI	26.1	29.2	28.6	25.7	31.1
% non-synchronous segregation	-	-	1.2±0.02	2.3±0.07	-
% Bridge	1.3±0.03	0.8±0.01	-	2.3±0.05	1.6±0.01
% Laggard	0.8±0.02	-	-	0.7±0.01	1.2±0.04
% Cytomixis	-	-	1.7±0.04	0.9±0.01	-
MII	95	87	108	79	82
% MII	13.4	11.5	14.4	10.4	11.2
% Sticky chromosome	4.3±0.08	4.8±0.03	2.1±0.02	1.5±0.07	1.3±0.02
% Cytomixis	-	-	1.2±0.08	-	-
AII/TII	315	332	321	348	303
% AII/TII	44.7	44.2	43	45.9	41.5
% Laggard	-	-	1.3±0.05	0.8±0.01	-
% Bridge	-	1.2±0.02	-	1.7±0.03	-
% Cytomixis	-	2.4±0.08	1.3±0.06	-	-
%non-synchronous segregation	0.6±0.01	-	-	-	
% micronucleus	1.3±0.08	-	0.8±0.01	1.5±0.06	0.5±0.01
% Tripolar	-	1.2±0.1	-	0.9±0.02	2.7±0.09
% Pentapolar	2.8±0.08	2.3±0.01	1.5±0.12	-	4.3±0.23

Abbreviations: D/MI = diakinesis/metaphase I; AI/TI = anaphase I/telophase I; MII = metaphase II; AII/TII = anaphase II/telophase II. All values are expressed as mean ± SE (standard error).

mother pollen cells. Chromosomes without orientation in the equatorial plane of the cell, also known as lagging or laggard chromosomes, are another abnormality that was observed in the anaphase I/telophase I stage in three species including *S. persepolitana*, *S. hypoleuca*, and *S. limbata* (Figure 1E). In the anaphase II/telophase II stages, only *S. sclarea* (Figure 1F) and *S. hypoleuca* showed this abnormality. Non-synchronous segregation of chromosomes is one of the meiotic abnormalities that may occur early or late. This abnormality was observed in all species. *S. sclarea* and *S. hypoleuca* exhibited this anomaly in meiosis I (with frequencies of 1.2±0.02 and 2.3±0.07, respectively), while *S. persepolitana* showed it only in meiosis II with a frequency of 0.6±0.01. Micronucleus is another meiotic abnormality that was observed in the tetrad stage in all studied species except *S. spinosa*. *S. hypoleuca* showed the highest frequency at 1.5±0.06 (Figure 1G), while *S. limbata* showed the lowest at 0.5±0.01. In the final stages of meiosis, tetrads with three (Figure 1H) and five poles (Figure 1I) are another abnormality that was observed in different frequencies in the studied species. No tripolar tetrads were observed in *S. persepolitana* and *S. sclarea*, and no pentapolar tetrads were observed in *S. hypoleuca*.

Meiotic index

The meiotic index, which is obtained by dividing the number of normal tetrads by the total number of observed tetrads and multiplying by 100, was reported to have the highest value in *S. persepolitana* and the lowest value *S. hypoleuca* among the studied species (Figure 2). The order of the meiotic index in the studied species was

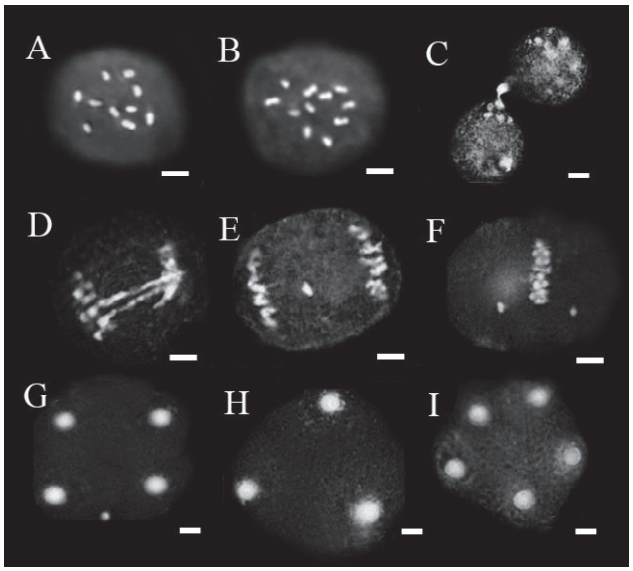

Figure 1. Representative meiotic cells in studied species of *S.* sect. *Aethiopis* (A-I). A: Diakinesis in *S. Spinosa* (showing 10 bivalents). B: Diakinesis in *S. sclarea* (showing 11 bivalents). C: Cytomixis in *S. hypoleuca*. D: Bridge in *S. limbata*. E: Laggards in *S. persepolitana*. F: Precocious separation in *S. sclarea*. G: Micronucleus in *S. hypoleuca*. H: Tripolar in *S. limbata*. I: Pentapolar in *S. spinose*. Scale bar: 3 µm.

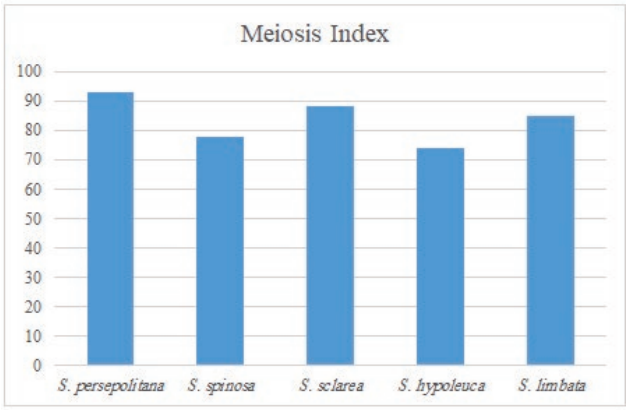

Figure 2. Comparison of Meiosis Index in studied species of *S.* sect. *Aethiopis.*

as follows: *S. persepolitana* > *S. sclarea* > *S. limbata* > *S. spinosa* > *S. hypoleuca*

Pollen fertility

Regarding pollen fertility, in the study of pollen grains in the *Salvia* genus, it was found that the fertility of pollen grains in all of them is above 80%. *S. persepolitana* showed the highest fertility (95%) and *S. spinosa*

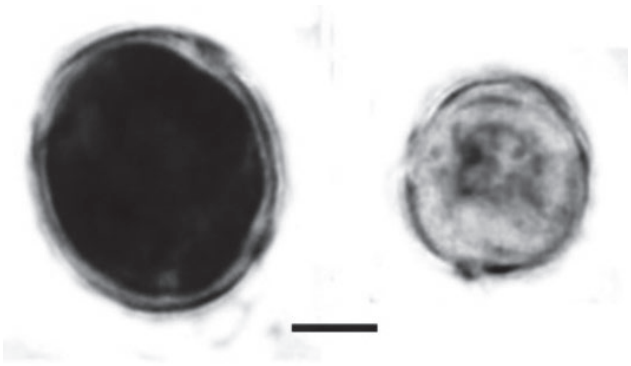

Figure 3. The fertile pollen is on the left side and the sterile pollen is on the right side. Scale bar: 5 µm.

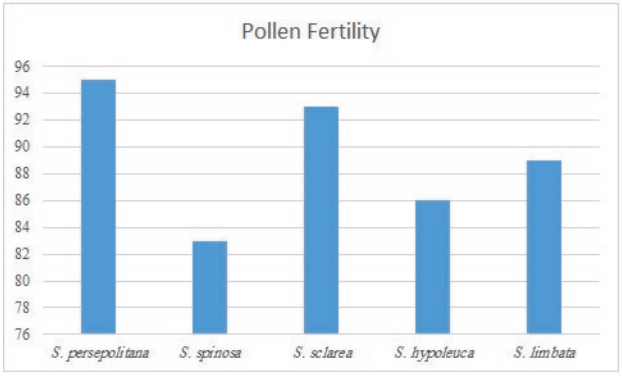

Figure 4. Comparison of pollen fertility in studied species of *S.* sect. *Aethiopis.*

showed the lowest fertility (83%) (Figures 3 and 4). The order of pollen fertility in different species is as follows: *S. persepolitana* > *S. sclarea* > *S. limbata* > *S. hypoleuca* > *S. spinosa*.

DISCUSSION

This study was conducted to investigate meiotic behavior, pollen fertility, and chromosomal abnormalities in five species of the genus *Salvia*. By studying the mother cells of pollen at the diakinesis stage, 10 and 11 bivalents were observed in the species under investigation. *S. persepolitana* and *S. spinosa* showed 2n = 20, which confirmed previous studies (Aryavand 1977; Kliphuis and Barkoudah 1977; Patudin et al. 1975; Afzal-Rafii 1981; Al-Turki et al. 2000; Sheidai et al. 2010; Ranjbar et al. 2015). *S. sclarea, S. hypoleuca, and S. limbata*, with 2n=22, also confirmed previous studies (Aryavand 1977; Afzal-Rafii 1980; 1981; Diaz et al. 1984; Rosúa and

Blanca 1988; Sheidai et al. 2010; Martin et al. 2011; Ranjbar et al. 2015). *Salvia* species have been reported to have varying chromosome numbers falling within different aneuploid series, inclung x = 6, 7, 8, 9, 10, 11, 13, 16 (Sheidai et al. 2010; Ranjbar 2015; Martin et al. 2022). Bahattacharya (1978) suggests that base numbers 7 and 8 are the primitive numbers from which secondary base numbers were established. These numbers then diversified in various directions, with base number 11 being common in many species. Martin et al. (2015) identified the chromosomal base number of 11 as a probable ancestral base number in the genus *Salvia*.

In examining the meiotic behavior and comparing the studied species, various types of chromosomal stickiness, cytomixis, lagging, and so on, with different frequencies, were observed. Among the studied species, *S. spinosa* showed the highest and *S. persepolitana* showed the lowest meiotic abnormalities in their PMCs.

Chromosomal stickiness

Chromosomal stickiness, which was observed in all the studied species with varying frequencies, was first observed in maize as chromatin clusters at the stage of pachytene (Beadle 1932). Sheidai et al. (2010) also reported the occurrence of this abnormality, either partially or completely, in all the studied species of the genus *Salvia* from the early stages of prophase to the final stages of meiosis. Ranjbar et al. (2015) reported chromosomal stickiness and chromosome bridges resulting from stickiness in some species of *Salvia*, including *S. hypoleuca* and *S. perseppolitana*. In this study, *S. hypoleuca* showed the highest percentage of chromosomal stickiness. Species with different evolutionary trajectories may have distinct mechanisms for chromosome segregation, potentially leading to variations in stickiness (Santos et al., 2017). Also, this abnormality is influenced by environmental and genetic factors (Pagliarini 2000).

Cytomixis

Chromosome displacement between PMCs, called cytomixis, was observed in different directions and at different stages of meiosis I and II in all the studied species, except for *S. perseppolitana*. The occurrence of cytomixis can be a cause of abnormal pollen grain formation. Ranjbar et al. (2015) reported this abnormality in *S. aethiopis* and *S. indica*. Kaur and Singhal (2019) reported this abnormality in *S. nubicola*. Alijanpoor and Safaeishakib (2022) mentioned the occurrence of cytomixis in *S. nemorosa*, *S. verticellata*, and *S. staminea*. The occurrence of cytomiixis can vary depending on the developmental stage of the plant. Factors such as cell differentiation, hormonal regulation, and tissue-specific gene expression may influence the propensity for cytomiixis in different plant species (Bhattacharya et al. 2016). Moreover, different species may encounter varying environmental factors, which can contribute to the diversity of cytomiixis abnormalities (Dutta and Chaudhuri, 2019).

Lagging chromosomes

Among the studied species, the highest frequency of lagging chromosome abnormality was observed in *S. hypoleuca*. Similar abnormalities have also been reported in other species of the genus *Salvia* (Sheidai et al. 2010; Ranjbar et al. 2015). In the study by Ranjbar et al. (2015), *S. hypoleuca* showed the highest frequency of lagging chromosomes. This abnormality was also reported in *S. nubicola* in another study (Kaur and Singhal 2019). The diversity of lagging chromosome abnormalities in studied species can be influenced by various factors. Differences in chromosomal organization among plant species and environmental factors may contribute to the diversity of lagging chromosome abnormalities (Kleckner 2006; Babu et al. 2016).

Bridges

Bridges, another abnormality, were observed in all the studied species except for *S. sclarea*, with different numbers and thicknesses. The overall incidence of this abnormality in meiosis I was almost twice that of meiosis II. In another study, while bridge abnormality was reported in *S. hypoleuca* in meiosis I, it was not observed in *S. perseppolitana* (Ranjbar et al. 2015). Kaur and Singhal (2019) also reported this abnormality in *S. nubicola*, and Alijanpoor and Safaeishakib (2022) reported it in several species of the genus *Salvia* such as *S. staminea*. Genetic and environmental factors are considered influential factors in these abnormalities and cause different frequencies in plant species. (Nirmala and Rao 1996).

Non-synchronous segregation

In the studied PMCs, both precocious and late separation were observed. This abnormality was not observed in *S. spinose* and S. *limbata* species. *S. hypoleuca* also showed this abnormality in the study by Ranjbar et al. (2015), while it was not observed in *S. persep-*

politana. Another study reported this abnormality in *S. nubicola* (Kaur and Singhal 2019). precocious separation of bivalents does not affect the normal separation of chromosomes, while late separation of chromosomes can lead to the formation of lagging chromosomes, chromatin bridges, and a decrease in pollen fertility (Kumar et al. 2010). Differences in chromosomal organization among plant species may contribute to the diversity of non-synchronous segregation abnormalities (Kleckner, 2006). Moreover, different plant species may encounter varying environmental factors, which can contribute to the diversity of non-synchronous segregation abnormalities (Babu et al., 2016).

Micronuclei

Micronuclei were a common abnormality in the studied species except for *S. spinose*. This abnormality was reported with a frequency of 50% in *S. limbata* in another study (Alijanpoor and Safaeishakib 2022). Laggards can contribute to the formation of micronuclei and may also result in aneuploid gametes, thereby playing a role in chromosomal evolution. Plant species with higher levels of genomic instability may exhibit a greater diversity of micronucleus abnormalities. Factors such as repetitive DNA content, transposable elements, and chromosomal rearrangements can contribute to genomic instability (Nagaki et al., 2012). Also, various environmental factors, such as radiation, chemical pollutants, and exposure to heavy metals, can induce micronucleus formation in plant cells (Jha et al. 2020).

Triad and pentad

In the studied species, the highest frequency of trisomy and pentasomy was reported in *S. limbata* and *S. persepolitana*, respectively. Such cells may lead to the formation of tetrad and abnormal and non-viable pollen grains. Bahattacharya (1978) reported triploid cells in two varieties of *S. splendens*. Sheidai et al. (2010) reported tripolar and multipolar cell formation due to anaphase I and II failure in *Salvia* species. Variations in genes involved in meiotic processes, such as synaptonemal complex formation, chromosome pairing, and cytokinesis, may lead to alterations in the formation of tetrads, resulting in triad or pentad abnormalities (Prieto et al. 2018). Different plant species may encounter varying environmental factors such as temperature, light intensity, nutrient availability, or exposure to stressors which can contribute to the diversity of triad and pentad abnormalities (Babu et al. 2016).

Overall, the frequency of meiosis abnormalities in studied species of *Salvia* sect. *Aethiopis* can vary due to factors such as genetic variation, environmental conditions, and evolutionary history. Genetic differences and chromosomal arrangements affect meiotic processes. The evolutionary history of a genus can also impact meiotic stability, with some species evolving mechanisms to reduce errors (Pawlowski and Cande 2005; Madlung 2013). Pagliarini (2000) suggested that anomalies affecting fertility can arise from mutations in genes controlling meiosis, with some abnormalities leading to complete male sterility in certain species. Variation in the activity of key meiotic genes (Caryl et al. 2003), recombination, chromosome synapsis, cell cycle control, chromosome distribution, and polyploidy (Mercier et al. 2015) can account for the varying levels of meiosis abnormalities observed among different species within a plant genus. The discrepancies in meiotic progression and recombination patterns between species can likely be attributed to variations in genome size and organization resulting from differences in repetitive DNA content and ploidy level (Lambing and Heckmann 2018). Natural variations in polymorphisms at the recombination site and specific DNA sequence motifs contribute to the variability in meiotic recombination frequency within and between species (Lawrence et al. 2017).

It's important to note that the specific research on meiosis abnormalities in plants may be limited. Further studies are needed to investigate the underlying mechanisms and factors contributing to the diversity of triad and pentad abnormalities in different plant species.

There is an inverse relationship between the meiotic index and chromosomal abnormalities during meiosis. Species that show the highest chromosomal abnormalities in their meiotic behavior have the lowest meiotic index and vice versa. In this study, *S. persepolitana* showed the highest meiotic index, and the total observed chromosomal abnormalities in this species were lower than in other studied species. *S. hypoleuca* had the lowest meiotic index with the highest frequency of meiotic abnormalities. According to Alberto et al. (2003), the degree of pollen grain stainability in the studied species of *Salvia* ranged from 75.7 to 97.6%. Sheidai et al. (2010), pointed out high pollen fertility (>0.90%) in their studied *Salvia* species.

CONCLUSIONS

Detailed cytological studies in species of *Salvia* sect. *Aethiopis* including *S. persepolitana*, *S. spinosa*, *S. sclarea*, *S. hypoleuca* and *S. limbata* showed diploid pro-

phase with two basic chromosomes (10 and 11). However, the examined species exhibited various chromosomal abnormalities, such as chromosomal stickiness, failures in normal separation during anaphase, cytomixis, and other anomalies, resulting in a decrease in pollen fertility. It is important to note that meiotic abnormalities do not always have detrimental effects and can contribute to the generation of genetic diversity. However, further research is necessary to gain a comprehensive understanding of these intricate interactions. Considering the numerous reports of varied chromosome numbers and ploidy levels in different species of the *Salvia* genus, such meiotic abnormalities may be a cause of the evolution of aneuploidy and polyploidy in this genus.

LIST OF ABBREVIATIONS

PMC: Pollen Mother Cell
MI: Meiotic Index

REFERENCES

Afzal-Rafii Z. 1980. In chromosome number reports LXVII. Taxon 29:365-366.

Afzal-Rafii Z. 1981. In chromosome number reports LXX. Taxon 30:73-74.

Alberto CM, Sanso AM, Xifreda CC. 2003. Chromosomal studies in species of *Salvia* (Lamiaceae) from Argentina. Bot J Linn Soc. 141:483-490.

Alijanpoor B, Safaeishakib M. 2022. Cytomixis and meiotic abnormal behavior related in some selected species of the genus *Salvia* L. (Lamiaceae) From Iran. ASM. 6(2):34-37.

Al-Turki TA, Filfilan SA, Mehmood SF. 2000. A cytological study of flowering plants from Saudi Arabia. Willdenowia 30:339-358.

Aryavand A. 1977. In IOPB chromosome number reports LVII. Taxon 26:443-452.

Babu P, Kalaiselvi M, Parani M. 2016. Effect of environmental mutagens on chromosomal abnormalities in plants. Plant Genome Diversity 2:189-207.

Bahattacharya S. 1978. Study of Some Indian Members of the Genus *Salvia* with References to the Cytological Behaviour. Cytologia 43:317-324.

Baptists-Giacomoelli FR, Pagliarini MS, Almeida JL. 2000. Elimination of micronuclei from microspores in a Brazilian oat (*Avena sativa* L.) variety. Genet Mol Biol. 23:681-684.

Beadle GW. 1932. A gene for sticky chromosomes in *Zea mays*. Zeitschr Ind Abst Vererbungsl. 3:195-217.

Bhattacharya S, Bandyopadhyay A, Das P. (2016). Cytomixis in plants: Fact or artifact? Cytol Genet. 50:274-282.

Boissier E. 1879. Flora Orientalis. Genevae et Basileae 4:823-854.

Caryl A, Jones G, Franklin F. 2003. Dissecting plant meiosis using *Arabidopsis thaliana* mutants. J Exp Bot. 54:25-38.

Celep F, Atalay Z, Dikmen F, Doğan M, Sytsma KJ, Claßen-Bockhoff R. 2020. Pollination ecology, specialization, and genetic isolation in sympatric bee-pollinated *Salvia* (Lamiaceae). Int J Plant Sci 181:800-811.

Defani-Scoarize MA, Pagliarini SM, Aguiar CG. 1995. Evaluation of meiotic behavior in double-cross maize hybrids and their parents. Maydica 40:319-324.

Diana Corrias S. 1983. Chromosome numbers for Flora Italian: Information Botanica Italiana, vol.15; p. 44-48.

Diaz Lifante Z, Luque T, 1992. Bárbara CS. Chromosome numbers of plants collected during Iter Mediterraneum II in Israel. Bocconea 3:229-250.

Dutta S, Chaudhuri S. 2019. Cytomixis: A potential source of variation. Plant Genome Diversity 2:103-118.

Epling C, Lewis H, Raven PH. 1962. Chromosomes of *Salvia*: Section *Audibertia*. Aliso. 5:217-221.

Eroğlu HE, Martin E, Kahraman A, Aslan EG. 2021. The new chromosomal data and karyotypic variations in genus *Salvia* L. (Lamiaceae): dysploidy, polyploidy and symmetrical karyotypes. Caryologia 74(4):21-28.

Haque MS. 1981. Chromosome numbers in the genus *Salvia* Linn. Proc Indian Acad Sci. 47:419-426.

Hedge IC. Labiatae. 1982a. In: Rechinger CH, editor. Flora Iranica. Akademische Druk-U. Verlagsanstalt, Graz, Austria 150:403-476.

Hedge IC. 1982b.Labiateae. In: Davis, P. H, editor. Flora of Turkey and the East Aegean Islands. Edinburgh University Press, Edinburgh, pp. 400-461.

Jha S, Chauhan PS, Sharma N. 2020. Genotoxicity and cytotoxicity of environmental pollutants in plant systems. In Environmental Pollution and Its Management. Springer, pp. 37-62.

Kaur D, Singhal VK. 2019. Meiotic abnormalities affect genetic constitution and pollen viability in dicots from Indian cold deserts. BMC Plant Biol. 19:1-11.

Kleckner N. 2006. Chiasma formation: chromatin/axis interplay and the role(s) of the synaptonemal complex. Chromosoma 115:175-194.

Kliphuis E, Barkoudah YI. 1977. Chromosome numbers in some *Syrian angiosperms*. Acta Bot Neerl. 26:239-249.

Kumar P, Singhal VK, Kaur D, Kaur S. 2010. Cytomixis and associated meiotic abnormalities affecting pollen fertility in *Clematis orientalis*. Plant Boil. 54:181-184.

Lambing C, Heckmann S. 2018. Tackling plant meiosis: from model research to crop improvement. Front Plant Sci. 9:1-19.

Lawrence E, Griffin C, Henderson I. 2017. Modification of meiotic recombination by natural variation in plants. J Exp Bot 68:5471-5483.

Madlung A. 2013. Polyploidy and its effect on evolutionary success: old questions revisited with new tools. Heredity 110:99-104.

Martin E, Celep F, Eroğlu HE. 2022. Comparative chromosomal features and new karyological data in *Salvia*: B-chromosomes, polyploidy, dysploidy and symmetric karyotypes, Braz J Bot 45:625-634.

Mercier R, Mézard C, Jenczewski E, Macaisne N, Grelon M. 2015. The molecular biology of meiosis in plants. Annu Rev Plant Biol 66:297-327.

Nagaki K. Tsujimoto H. Sasakuma T. (2012). Unstable chromosome behavior at mitosis and cytokinesis in plants. Cytogenet Genome Res. 136:205-214.

Pagliarini MS. 1990. Meiotic behavior and pollen fertility in *Aptenia cordifolia* (Aizoaceae). Caryologia 43:157-162.

Pagliarini MS. 2000. Meiotic behaviour of economically important plant species: the relationship between fertility and male sterility. Genet Mol Biol. 23:997-1002.

Patudin AV, Yurtsev VN, Pakaln DA. 1975. Chromosome number in some species of *Salvia* L. (Lamiaceae). Bot Zh. 60:529-534.

Pawlowski WP, Cande WZ. 2005. Coordinating the events of the meiotic prophase. Trends Cell Biol. 15:674-681.

Prieto P. Shaw P. Moore G. 2018. Homologue recognition during meiosis is associated with a change in chromatin conformation. Nat Commun. 9:1-10.

Ranjbar M., Pakatchi A., Babataheri Z. 2015. Chromosome number evolution, biogeography and phylogenetic relationships in *Salvia* (Lamiaceae). Webbia 70:55-62.

Rosúa JL, Blanca G. 1988. Revisión del género *Salvia* L. sect. *Aethiopis* Benth. (Lamiaceae) en el Mediterráneo occidental. Collect Bot. 17:205-236.

Sheidai M, Alijanpoo B, Khayyami M. 2010. Contribution to cytology of genus *Salvia* L. (Lamiaceae) in Iran. Caryologia 63:405-410.

Sheidai M, Alijanpoo B, Khayyami M. 2022. Some selected species of the genus *Salvia* L. (Lamiaceae) from Iran. ASM. 6:34-37.

Soodan AS, Wafai BA. 1987. Spontaneous occurrence of cytomixis during Microsporogenesis in almond (*Prunus amygdalus* Batsch). Cytologia 52:361-364.

Standley P, Williams L. 1973. Labiatae. Fieldiana Bot. 24:237-317.

Santos AP, Ferreira WM, Techio VH. 2017. Chromosome stickiness and nuclear abnormalities in plants: a review. Caryologia 70:190-198.

Tedesco SB, Schifino-Wittmann MT, Dall'Agnol M. 2002. Meiotic behaviour and pollen fertility in the seventeen Brazilian species of *Adesmia* DC. (Leguminosae). Caryologia 55:341-347.

Tantray YR, Jan I, Wani MS, Singhal VK, Gupta RC. 2021. Chromosome numbers and meiotic behavior in some species of Asteraceae from high altitudinal regions of Kashmir Himalayas. J Asia-Pac Biodivers. 14:590-606.

Utsunomiya KS, Bione NCP, Pagliarini MS. 2002. How many different kinds of meiotic abnormalities could be found in a unique endogamous maize plant? Cytologia 67:169-176.

Vij SP, Kashyap SK. 1975. In IOPB chromosome number reports XLVIII. Taxon 24:367-372.

Wu CY, Li HW. 1982. On the evolution and distribution in Labiateae. Acta Bot Hung. 4:97-118.

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An account of chromosomal damage in PMCs of stripe rust infected barley

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Abstract. A study, on the effects of natural mild fungal infection on meiosis of Barley Pollen Mother Cells (PMCs), was done in order to analyze the chromosomal damage elicited by the pathogenic conditions. A pattern similar to common mutagens, of reduction in mitotic index and chiasma frequency, as well as, production of various aberrations that demonstrate chromosomal damage, was observed. The most common abnormalities were un-orientation and other spindle related aberrations, as well as stickiness and clumping of chromosomes. The disease induced a reduction in pollen viability as compared to the control plants. The results were compared with those of a high dose of a known mutagen ie gamma rays in order to draw commonalities between the two conditions.

Keywords: anomalies, Barley, chromosomes, fungal mycotoxins, gamma rays, *Puccinia*.

INTRODUCTION

Barley (*Hordeum vulgare* L.) is among the most important cereal crops in the world. In India, it is used for the purposes of animal feed, flour making and for malting and brewing purposes (Selvakumar et al., 2014; Singh et al., 2019). Barley is a low input crop and has much better adaptability when compared to wheat (Verma et al., 2012). Barley is naturally inbred and provides a very good genetic material for study of mutagenesis using various agents like radiations, chemicals or combinations of both. The response of this crop in transferring the mutations from one generation to another is exceptionally good which makes it a preferred choice of material for mutagenic studies.

This important crop suffers from various diseases causing great reduction in yield and grain quality. One such disease is the stripe rust caused by *Puccinia striiformis* f. sp. *hordei* (Psh). This disease is common in various countries of South Asia, East Africa, and Central and North America. *P.striiformis* f. sp. *hordei* is a macrocyclic rust having two hosts, primary host being Barley. The most damaging spore, in this fungus, is the uredospore which follows multiple asexual cycles to spread the disease. The primary symptoms include yellow/orange pustules (uredosorus) lined linearly along midribs. The diseased plants are shorter, less vigorous and have a poor root

system. The photosynthesis is affected as a result of the dark pustules reducing the green area. Grains are poorly filled and many florets show abortion. It affects both quantity and quality of grain production (Luthra and Chopra, 1990; Roelfs and Huerta-Espino, 1994).

All exogenous agents capable of producing chromosome aberrations (CAs), i.e., clastogens, are mutagens, and most are also carcinogens. For that reason, cytogenetic damage has long been a favored surrogate endpoint for assessment of carcinogenic and mutagenic potential. One very important conclusion from ionizing radiation (IR) mutagenesis studies in cells of higher organisms has been that large-scale genomic structural changes generally dominate the spectrum of new mutations, as compared with point mutations or other small intragenic changes. Granted, the spectrum of mutations can differ vastly, depending on the mutagenic agent, but in the present study, our emphasis will be on mutagenic events resulting from large-scale structural changes to the host chromosomes genome caused by fungal pathogenesis. These include deletions, insertions, inversions, and translocations, any of which can disrupt genes, alter the control of gene expression, or even result in expression of new fusion sequences. IR is virtually unique regarding its efficiency for producing prompt DNA double-strand breaks (DSBs) randomly throughout the genome, which is the prerequisite lesion for the development of these structural rearrangements (Cornforth et al, 2021). An analogy of the action of IR with fungal toxin will thus enable us to understand the mechanism by which pathogen brings about chromosomal damage and heritable changes in host.

It can be speculated that together with physiological effects, the pathogens, like fungus, might have some cytogenetic effects on the host plant. Though a number of studies attributing the effect of fungal toxins on inhibition of various enzymes and interference with physiological processes are available the effect at cellular and genetic level has not been explored. The level of DNA damage after treatment with fungal metabolites would be related to the ability of the host to survive and reproduce after infection. Impaired activity of antioxidant defense and DNA repair contribute to the DNA damage by free radicals. A few workers have observed induction of chromosomal anomalies by fungal infections eg *Aspergillus* on Cotton (El-Naghy, 1992), *Fusarium* on wheat and maize (Helmey, 2003), *Fusarium* on Maize (El-Daisty, 2009) etc. Various studies have evaluated the effects of radiations, chemicals, pesticides, plant metabolites etc on the genetic material for the purpose of mutagenesis but very few studies are available which show the impact of microbial toxins up to the Chromosomal/DNA level (Kaur et al, 2018). Therefore, it was planned to study the meiosis in fungus infected plants and compare it with a known mutagen ie Gamma radiations, in order to evaluate the chromotoxic potential of fungal toxins.

MATERIALS AND METHODS

Hordeum vugare variety K10 of barley was used for the study. Naturally infected plants were monitored for morphological parameters and young ears collected at the time of flowering. At the flowering time, ie about 50 days after planting, floral buds were collected and fixed in Farmer's fixative (3:1 absolute ethanol-acetic acid) for 24 h. They were then transferred to 70% alcohol and stored at 4°C. Cytological investigations were done using 1% acetocarmine squash technique. Anaphase and Metaphase stages were considered as active division. All chromosomal abnormalities were screened and recorded under the respective stages of cell division where they occurred. Pollen viability was estimated by Acetocarmine stain method where deeply stained pollen grains were considered viable, while non-stained ones were considered non-viable. Similarly, gamma irradiated sets of half of LD_{50} ie 25 kR were screened for comparison of all parameters. Suitable controls were also maintained and all sets given exactly similar environmental conditions.

RESULTS AND DISCUSSION

The control buds showed perfect bivalents at metaphase I and a separation of 7:7 at anaphase I. Metaphase II and Anaphase II were also perfectly normal in controls. However the fungal infected sets showed various types of abnormalities (Fig. 1). The total abnormality percentage was moderate. Common Metaphase anomalies included stickiness, clumping, precocious movement, fragmentation, multivalent formation, univalents, secondary association, unorientation etc. The Anaphase was also marked with different types of chromosomal anomalies like stickiness, laggards, bridges, unequal separation, multipolarity and micronuclei. Table 1 presents a list of anomalies induced by fungi as well as those induced by gamma rays, on the meiosis of barley.

Meiotic anomalies have been reported by a number of workers in a variety of crops following mutagenic treatments eg Wani & Anis, 2008 (Gamma rays on *Cicer*), Pakorn etal, 2009 (Gamma rays on *Anubias*), Motilal etal, 2012 (EMS on *Astercantha*), Akhtar, 2014

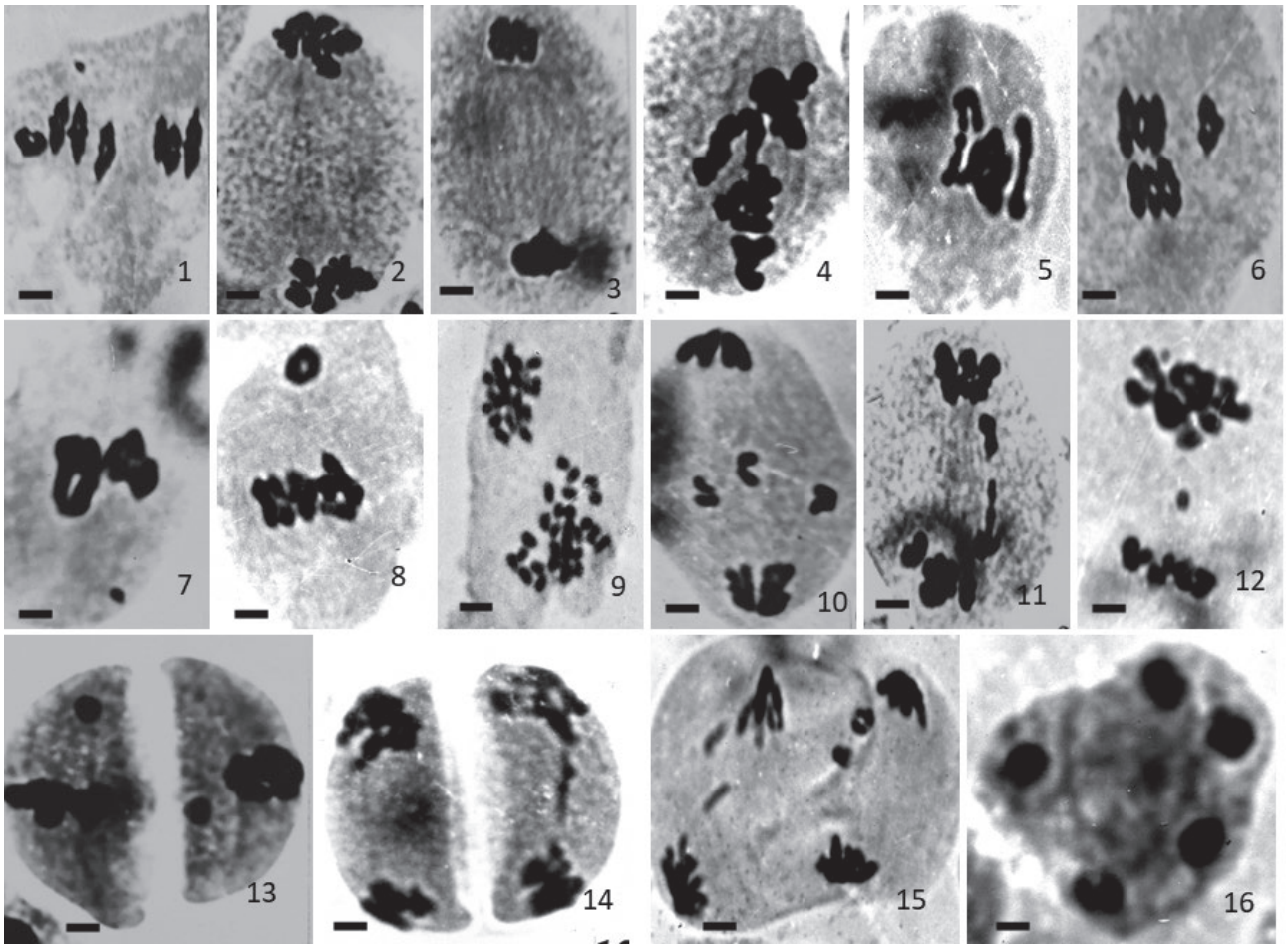

Figure 1. Cytological anomalies induced by chromosomal damage in Barley; 1- Normal Metaphase I, 2 - Normal Anaphase I showing 7:7 separation, 3 – Normal Telophase I, 4 – Stickiness and secondary association of bivalents at Metaphase I, 5 – Multivalents, 6 – Unorientation at Metaphase I, 7 – Clumped multivalents, 8 – Precocious movement, 9 – Fragmentation, 10 – Laggards at Anaphase I, 11 – Chromosome bridge, 12 – Unequal separation and laggard at Anaphase I, 13 – Precocious movement at Metaphase II, 14 – Bridge at Anaphase II, 15 – Laggards at Anaphase II, 16 – Multipolarity at Telophase II. [Scale Bar 1 cm = 4μ].

(Gamma rays and EMS on *Solanum*), Asare etal, 2017 (Gamma rays on *Abelmoschus*), Gnankambary etal, 2019 (Gamma rays on *Vigna*), Chen etal, 2020 (EMS on *Arachis*), Rashid etal, 2021 (Stress on *Trillium*), Liu et al, 2022 (Natural factors on *Elymus*), Turkoglu etal, 2023 (Sodium Azide on *Triticum*) etc. However there are only a few studies which suggest that fungal toxins may also induce chromosomal anomalies. Agar and Alpsoy (2005) studied aflatoxin G_1 (AFG_1) induced chromosomal aberrations in *Vicia faba* and *Zea mays*. Their results showed that 0.1, 0.2, 0.4 ppm concentrations of aflatoxin G_1 could induce gradient based chromosomal aberrations.

Since, they point towards the instability of the genome, chromosomal damages and aberrations have often been regarded as the index of cytotoxic potential of a mutagen. As far as radiations and chemical mutagens are concerned, an increase in cytological anomalies is the obvious manifestation. But similar effects induced by mycotoxins, is an interesting aspect of this study.

Different authors have given various explanations for occurrence of different anomalies. In general the metaphase abnormalities are related to spindle dysfunction e.g. scattering, unorientation and precocious movement of chromosomes. An alteration in genes governing spindle formation may lead to a loss of directive influence on chromosome arrangement and movement leading to consequent dysfunctional anomalies. The current observations in fungal treatment are in concurrence with studies of Styer and Horace (1984). They treated maize roots with solutions of moniliformin (a metabolite of *Fusarium moniliforme* Sheldon). They mentioned that higher concentration caused a disruption of the spindle apparatus.

Table 1. A comparison of cytological abnormalities induced by gamma rays and fungal infection in meiosis of barley.

Treatments	CF/biv ± SE	Metaphase I/II abnormalities (%)								Anaphase I/II abnormalities (%)						Telophase I/II abn Cytoki-nesis (%)				abn (%)	TAb (%) ± SE
		Lm	Do	Pc	Mv	Uv	Fg	St	Cl	Sa	Lg	Br	Us	Ns	St	Mp	Lg	Br	Mn	Tr	Pa		
Control	1.66 ± 0.05	0.07	0.13																			0.20 ± 0.11
Gamma Rays (20 kR)	1.48 ± 0.06	0.61	0.67	0.33	0.27	0.20		2.03	0.54	1.55	1.01	0.88		0.82	1.21		0.40	0.10	0.81	0.11		11.54 ± 0.23
Fungal infected	1.30 ± 0.10	0.95	0.36	0.68	0.31	0.29	0.27	1.26	0.82	0.22	1.97	0.77	0.54		1.04	0.47	0.54		1.02		0.41	11.92 ± 0.18

Lm=Late movement of bivalents; Do=Disturbed orientation of chromosomes; Pc=Precocious movement of chromosomes; Mv=Multivalent formation; Uv= Univalent formation; Fg=Fragmentation of chromosomes; St=Stickiness of chromosomes; Cl=Clumping of chromosomes: Sa=Secondary association of bivalents; Lg=Lagging chromosomes; Br=Bridge formation between poles; Us=Unequal separation of chromosomes at anaphase; Ns=Non synchronous disjunction; Mp=Multipolarity; Mn=Micronuclei; Tr=Triads; Pa=Polyads; TAb=Total Abnormality; SE=Standard Error of Mean.

The presence of univalents and multivalents at metaphase has been reported in different mutagenic studies. Multivalent formation could be attributed to irregular pairing and breakage followed by translocations and inversions. The predominance of ring or chain multivalents is dependent upon the length of interchanged segments and position of interchange. Stray bivalents at metaphase I and II are usually caused by spindle disfunction (Bhat et al., 2007b). The observed precocious chromosomes migration to the poles may be resulted from univalent chromosomes at the end of prophase I or precocious chiasma terminalization at diakinesis or metaphase I. Precocious migration of univalents to the poles is found to be a very common abnormality among plants which have been treated with mutagens (Consolaro et al., 1996). Secondary associations can result from modified chromosomes arrangement due to the duplication, interchanges or stickiness (Kumar and Singh 2003).

Chromosome stickiness has been reported to be a result of partial dissociation of the nucleoprotein and alteration in their pattern of organization (Evans 1962). Mc Gill et al (1974) and Klasterska et al (1976) suggested stickiness due to improper folding of chromosome fibre. Jayabalan and Rao (1987) reported stickiness in meiosis as due to the disturbances in cytochemically-balanced reactions by secondary effects of radiations.

Fragmentations or chromosome shattering observed in present study has also been reported by Cremer and Cremer (1986), Albanese (1982), Cremer et al (1981) as effects of radiation alone or in combination with chemicals. These may be due to damaged mechanisms of DNA repair caused by radiations (Periera, 1995).

Laggards were one of the most common Anaphasic abnormalities characterized by delayed movement of some chromosomes during Anaphasic separations. These have been reported by a number of workers and may be due to delayed terminalization, stickiness of chromosomes ends or because of failure of chromosome movement (Permjit and Grover 1985, Jayabalan and Rao 1987, Sohair 1989). These laggards may move randomly to any pole and give rise to unequal separation of chromosomes or they may form a pole by aggregating together and causing multipolarity. These may just clump together while remaining away from daughter nuclei at each pole and form micronuclei at Telophase.

Bridges are also a very common chromosome damage indicator. These are caused by paracentric inversion, which lead to formation of a dicentric bridge joining two poles (Swanson, 1988). The bridges may also be formed by stickiness between separating chromosomes. During separation these bridges break randomly and give rise to unbalanced poles having unequal chromatin volume.

Disturbances in spindle formation in meiosis II leads to formation of three or more than four poles at Ana/Telophase II. Subsequent wall formation gives rise to triads or polyads instead of normal isobilateral tetrads.

Changes in the surface proteins of PMC walls, lead to clumping of PMCs and sometimes gives rise to cytoplasmic channels allowing transmigration of chromatin. This is known as cytomixis and it is a powerful agent in causing polyplodization and increase in chromosome numbers within PMCs. Changes in cytoplasmic viscosity may also lead to shrinkage of PMCs, which was evident in a few PMCs.

The chiasma frequency showed a decrease in fungi infected plants. Presence of greater number of univalents might be responsible for a consequent decrease in the chiasma frequency although it may get balanced somewhat by a simultaneous increase in multivalents. Greater occurrence of rod bivalents might also cause a decrease in chiasma frequency. Some authors like Raghuvanshi and Singh (1974) have reported a decrease in chiasma frequency with increase in dose of treatment while some others like Prasad and Godward (1969) had observed an opposite trend. The reduction observed here is common to most radiation and chemical mutagenic treatments and has been demonstrated by workers like Sinha and Mahapatra (1969) in *Zea,* Sinha and Roy (1976) in *Phaseolus* and Lal and Srinivasachar (1979) in *Pennisetum*.

A high degree of pollen sterility, in gamma treatment as well as fungi infected sets, is a result of increase in the chromosomal abnormalities, which give rise to pollen with varying degrees of chromosomal imbalance. Pollen sterility has been attributed to stickiness that leads to irregular segregation and improper fragmentation of chromosomes. Such unbalanced pollen grains are very often non-viable and unable to fertilize the ovules. This in turn causes adverse impact on seed setting.

A comparison of the chromosomal anomalies present in Fungi infected plants with those present in conventional mutagens like gamma irradiated or chemical treated plants reveals a great level of similarity. When we compare the results obtained with by fungal pathogens with those elicited by other mutagens, we get striking similarities which indicates similar mode of action. Kumar and Yadav (2010) reported almost similar chromosomal anomalies induced in *Sesamum indicum* (L.) by EMS (Ethyl Methane Sulphonate) which is an alkylating agent. Singh et.al (2019) and Nilan et.al (1964) also found identical chromosomal damage was reported by use of radiations. Studies suggest that even some non-conventional agents like Catalase and Lipase enzymes have elicited reduction in germination and survival of plants (Ananthaswamy et.al; 1971). However, if fungal pathogen induced mutations are considered, there was a clear predominance of physiological abnormalities like stickiness and clumping over clastogenic ones like fragmentation or micronuclei. Such anomalies lead to high degree of gamete sterility and bring the plant into a growth disadvantage. As a result high degree of lethality is induced even at low infections.

It seems that the reduction in active mitotic division occurs due interference of chemicals in the G1 cell cycle which suppresses DNA synthesis as reported by Mohandas and Grant (1972) in several higher plants. There are many studies that compare the chromosomal abnormalities induced by the chemical, physical mutagens and the combination of both like those of Sree Ramalu (1973), Mehra and Mann (1974), Kumar and Singh (2002), Alam et al(2022) etc. However, the progress in the effective and efficient use of mutagens is hindered by complex interplay of many physical and chemical factors that determine the ultimate yield of mutations (Konzak et al 1975). According to Wilson (2019) and Jeong (2014) ionizing radiations can stimulate ROS production through nitric oxide synthase (NO) pathway. Interaction of NO molecule with superoxide radical (O_2^-) to produce peroynitrite ($ONOO^-$). Peroxynitrite is a powerful oxidant radical reacts with DNA bases, amino acids and lipids. NADPH oxidase is also been reported to cause production of ROS. When the ROS encounter biological organisms, they cause damage to biomolecules such as DNA, RNA and proteins in living cells.

It is evident that fungal toxins either themselves act as mutagens or induce formations of certain chemicals in the host which causes chromosome damage. DNA damage during plant interactions with virulent pathogens is largely under-described, and whether DNA damage arises during responses activated by core plant defense mediators such as salicylic acid, jasmonic acid or activated microbe-associated molecular pattern (MAMP) receptors also is not known (Song and Bent 2014). The present study calls for a calibration study on chromosomal damage by mycotoxins which can have intergenerational effects. It also brings into forefront that fungal diseases can have manifestations that are not only physiological but may also be genetical. Damaged genes may bring about mutations, at least some of which may show some degree of inheritance. A deeper study in this area is required.

REFERENCES

1. Agar, G. and L. Alpsoy (2005). Antagonistic effect of selenium against aflatoxin G1 toxicity. Botanical Bulletin of Academia Sinica 46:301-305.

2. Akhtar, N. (2014). Effect of physical and chemical mutagens on morphological behavior of tomato (*Solanum lycopersicum*) cv. "Rio grande" under heat stress conditions. Plant Breeding and Seed Sci. 70: 69-79.
3. Alam Q., Khan M.A. and Azaz Ahmad Z.R. (2022). Gamma Irradiation Induced Chromosome Aberrations in Meiotic Cells of Bread Wheat (*Triticum aestivum* L.). Int. J. Plant and Soil Sc. 34(5):81-89.
4. Albanese, R. (1982). The use of fertilized mouse eggs in detecting potential clastogens. Mutat. Res. 97:315-326.
5. Ananthaswamy H. N., Vakil U.K., Sreenivasan A. (1971). Biochemical and physiological changes in gamma-irradiated wheat during germination. Radiation Botany 11:1-12
6. Asare, A. T., Mensah, F., Acheampong, S., Asare-Bediako, E., and Armah, J. (2017). Effects of gamma irradiation on agromorphological characteristics of okra (*Abelmoschus esculentus* L. Moench.). Adv. Agric. 2017(3):1–7.
7. Bhat, T. A.; M. Sharma and M. Anis (2007b). Comparative Analysis of Meiotic Aberrations Induced by Diethylsulphate and Sodium Azide in Broad Bean (*Vicia faba* L.). Asian J. Plant Sciences, 6 (7):1051-1057.
8. Chen, T., Huang, L., Wang, M., Huang, Y., Zeng, R., Wang, X. (2020). Ethyl methyl sulfonate-induced mutagenesis and its effects on peanut agronomic, yield and quality traits. Agronomy 10(5):655-667.
9. Consolaro, M. E. L. and Pagliarini, M. S. (1996). Spontaneous Chromosome Stickiness in Microsporocytes of *Centella asiatica* (L.) Urban (Umbelliferae), Cytologia 61:57-61.
10. Cornforth M.N., Bedford J.S. and Bailey S.M. (2021). Radiation on Chromosomes: Sizing up the Damage. Cytogenet Genome Res 161(6-7):328–351.
11. Cremer, C. and Cremer, T. (1986). Induction of chromosome shattering by UV light and Caffeine: The influence of different distributions of photolesions. Mut. Res. 163:33-40.
12. Cremer, C., Cremer, T., Zorn, C. and Zimer, J. (1981). Induction of chromosome shattering by UV irradiation and caffeine: Comparison of whole-cell and partial cell radiation. Mut. Res. 84:331-348.
13. El-Diasty, Z. M., Zaied, K.A., Kosba, Z.A., Kash, K. S. and Abd El-Azez, M. H. (2009). Meiotic chromosomal anomalies resulting from fungal infection of maize compared with those resulting from gamma irradiation. J. Agric. Sci. Mansoura Univ., 34(9):9233-9248.
14. El-Naghy, M.A., Mazen, M.B. and Fadl-Allah, E.M. (1991). Studies on the fungus flora and aflatoxin production of cotton seeds in Egypt. Medical Journal of Islamic World Academy of Sciences. 4(2):141-145.
15. Evans, H. J. (1962). Chromosomal aberration induced ionizing radiations. Int. Rev. Cytol. 13:221-232
16. Gnankambary, K., Batieno, T. B. J., Sawadogo, N., Sawadogo, M., Yonli, D., and Ouédraogo, J. T. (2019). Assessment of radio-sensitivity for three cowpea genotypes to gamma irradiation. Int. J. Genet. Mol. Biol. 11(2):29-33.
17. Helmey, R. K. (2003). Cytological studies of some Fusarium toxins on growth, cell ultrastructure and electrophoretic profile of seed proteins in wheat and maize. Ph. D Thesis, Botany Department, Faculty of Science, Minia University, Minia, Egypt.
18. Jayabalan, N. and Rao, G. R. (1987). Gamma radiation induced cytological abnormalities in *Lycopersicon esculentum* Mill var. Pusa Ruby. Cytologia 52:1-4.
19. Jeong K. S. (2014). Effects of temperature-dependent optical properties on the fluence rate and temperature of biological tissue during low-level laser therapy. Lasers in Medical Science 29(2):637-644.
20. Kaur M., Chadha P., Kaur S., Kaur A., Kaur K., Yadav A. K., and Kaur R. (2018). Schizophyllum commune induced genotoxic and cytotoxic effects in Spodoptera litura. Scientific Reports 8:4693.
21. Klasterska, I., Natarajn, A.T. and Ramel. C. (1976). An Interpretation of the origin of sub-chromatid aberrations and chromosome stickiness as a category of chromatid aberration. Hereditas 83:153-162.
22. Konzak C.F., Niknejad M., Wickham I. and Donaldson E. (1975). Mutagenic interaction of sodium azide on mutations induced in barley seeds treated with diethyl sulfate or *N*-methyl-*N*'-nitrosourea. Mut. Res. 30(1):55-61.
23. Kumar G. and Yadav. R.S. (2010). EMS induced genomic disorders in sesame (Sesamum indicum l.) Rom. J. Biol. – Plant Biol. 55(2):97-104.
24. Kumar, G and Singh, V. (2003). Comparative analysis of meiotic abnormalities induced by gamma rays and EMS in barley. J. Ind. Bot. Soc. 82:19-22.
25. Liu B., Tao X., and Dou Q (2022). Meiotic chromosomal abnormality detected in heterozygotes of *Elymus nutans*. Front. Plant Sci 13:3-9.
26. Luthra, J. K. And Chopra, V. L. (1990). "Genetics of stripe rust resistance in barley", Ind. J. Genet. Plant Breeding 50(4):390-395.
27. Mc Gill, M., Pathak, S. and Hsu, T.C. (1974). Effect of Ethidium bromide on mitosis and chromosomes: A possible amatorial basis for chromosomal stickiness. Chromosoma 47:157-168.
28. Mehra, P. N. and Mann, A. C. (1974) Cytological effect of chemical mutagens on Pterotheca falconeria

1. Monofunctional alkylating agent. Nucleus 17:167-182
29. Mohandas, T and Grant, W. F. (1972). Cytogenetic effects of 2,4-D and amitrole in relation to nuclear volume and DNA content in some higher plants. - Can. J . Genet. Cytol. 14:773-783.
30. Motilal B., Jogeshwar, P., Mishra, R. R. and Rath, S. P. (2012). Analysis of EMS induced in vitro mutants of *Asteracantha longifolia* L. Nees using RAPD markers. Indian J. Biotechnology 11:39-47.
31. Nilan. R. A., Konzac, C. F., Heiner, R. E. and Froese-Gertzen, E. E. (1964) Chemical Mutagenesis in barley. Proc. In: Int Barley Genetics Symp.Washington pp. 35-54.
32. Pakorn, T., Taychasinpitak, T. Jompuk C. and Jompuk. P. (2009). Effects of Acute and Chronic Gamma irradiations on in vitro culture of *Anubias congensis*. J. Nat. Sci. 43:449-457.
33. Pereira, C. M., Taschetto, O. M., Defani-Scoarize, M. A. and Pagliarini, M. S. (1995). Spontaneous Chromosome Fragmentation in Maize Microsporocytes. Cytologia 60:297-301.
34. Permijit, K. and Grover, I. S. (1985). Cytological effects of some organophosphorus pesticides II. Meiotic effects. Cytologia 50:199-211.
35. Prasad, A. B. and Godward, M. B. E. (1969). Comparison of the developmental response of diploid and tetraploid *Phalaris* following irradiation. Rad. Bot 9:167-173.
36. Raghuvanshi, S.S. and Singh, A.K. (1974). Studies on effect of gamma rays on *Trigonella foenum graecum* L. Cytologia 39:473-482.
37. Rashid, K., Rashid, S., Ganie, A. H., Nawchoo, I. A., Khuroo, A. A. (2021). Meiotic Studies, Pollen Fertility and Seed Set of *Trillium govanianum*, an Endangered Endemic Plant Species of the Himalaya. Cytologia 86:245-249.
38. Roelfs, A.P. and Huerta-Espino, J. (1994). Seedling resistance in *Hordeum vulgare* to barley stripe rust from Texas. Plant Dis 78:1046-1049.
39. Selvakumar, R., Meeta, Madhu, Shekhawat, P.S., Verma, R.P.S. and Sharma, I. (2014). Management of stripe rust of barley using fungicides. Indian Phytopath. 67(2):138-142.
40. Singh, B., Mehta, S., Aggarwal, S. K. and Tiwari, M. (2019). Barley, Disease Resistance, and Molecular Breeding Approaches. In book: Disease Resistance in Crop Plants. Edition I, Springer, Cham; pp. 261-299.
41. Sinha S.K. & Mohapatra B.K., (1969). Compensatory chiasma formation in maize. Cytologia 34:523–527.
42. Sinha, S. S. N. & Roy. H. (1976). Distribution of chiasmata between and within nuclei in irradiated and non- irradiated population in Phaseolus sp. J. Cytol. Genet. 11:7-9.
43. Sohair, E. Antoinette, H. and Atif, H. (1989) Cytogenetic effects of herbicide Garlon 4 on root mitosis of *Allium cepa*. Cytologia 54:465-472.
44. Song, J. and Andrew, A. F. (2014). Microbial Pathogens Trigger Host DNA Double-Strand Breaks Whose Abundance Is Reduced by Plant Defense Responses. Plos Pathogens 10(6):1-16.
45. Sree Ramalu, K. (1973). Mutagenic effects of gamma rays, chemical mutagens and Combined treatments in Sorghum. P. Pfanzucitg. 68:287-293.
46. Srinivasachar H.R. (1979). Induction of segmental interchanges in pearl millet (Pennisetum typhoides). Theoretical and Applied Genetics 54(1):27-34.
47. Styer, C. H. and. Horace, G. C. (1984). Effects of Moniliformin on Mitosis in Maize (*Zea mays* L.). Plant and Cell Physiology 25(6):1077-1082.
48. Swanson, C. P. (1988). Variation: Nature and Consequences of Altered Chromosomal Structure. Cytogenetics, 2nd ed., Prentice Hall of India.
49. Turkoglu, A., Haliloglu, K., Tosun, M., Szulc, P., Demirel, F., Eren, B., Bujak, H., Karagoz, H., Selvet, M., Uzkan, G. and Niedbala, G. (2023). Sodium Azide as a Chemical Mutagen in Wheat (*Triticum aestivum* L.): Patterns of the Genetic and Epigenetic Effects with iPBS and CRED-iPBS Techniques. Agriculture 13(6):1242-1259.
50. Verma, R.P., Sharma, R.K. and Mishra, B. (2012). Barley Cultivars released in India. Names, Parentages, Origins and adaptations, Research Bulletin No. 29. Directorate of Wheat Research, Karnal.
51. Wani, A., and Anis, M. (2008). Gamma ray- and EMS-induced bold-seeded high-yielding mutants in chickpea (Cicer arietinum L.). T. J. Biol. 32:161-166.
52. Wilson A, Menon V, Khan Z, Alam A, Litovchick L, Yakovlev V. (2019). Nitric oxide-donor/PARP-inhibitor combination: A new approach for sensitization to ionizing radiation. Redox Biology 24:101-169.

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Role of ten different exogenous plant growth promoters in regulating cytotoxic and genotoxic processes in barley exposed to high temperature stress

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Abstract. Agricultural crop affected preliminary and the most prominent by the adverse effects of global climate change have to adapt to various abiotic factors that will occur as a result of climate fluctuations in the near-future and struggle to survive. Among abiotic factors, the one of the greatest impact on plant stress is high temperature. Therefore, the most important step to take action against the global threat is the development of new temperature tolerant varieties. Barley, which is the fourth most important cereal in the world after wheat, maize, and rice are affected by high ambient temperatures. In this work, the effects of their alone and in double, triple combinations of ten various plant growth regulators (PGRs) on mitotic activity and chromosome behaviors in root meristems of barley exposed to high temperature (30°C) were investigated. In the experiments, *Hordeum vulgare* L. cv. Bülbül 89 variety and GA_3, KIN, BA, E, EBR, TRIA and PAs (Spm, Spd, Put, Cad) as growth regulators were used. The results obtained were compared with each other and with those of the seeds germinated at optimum temperature (20°C). Consequently; it has been determined that most of the PGRs studied, especially the GA_3 and their combinations with GA_3, exhibit a very successful performance on mitotic activity and cytogenetic aberrations in barley seeds germinated under high temperature stress- HTS conditions. The effects of these PGRs (except for EBR) and their combinations on mitotic activity and chromosome behaviors under HTS have been presented in this study for the first time.

Keywords: chromosomal aberrations, heat stress, *Hordeum vulgare* L., mitotic index, plant growth regulators.

INTRODUCTION

Climate change is an inevitable phenomenon globally, which affects all aspects mankind, including agricultural production worldwide today. According to IPCC report (2021) projects that in the coming decades climate changes will increase in all regions and for 1.5°C of global warming there will be increasing heat waves- longer warm seasons and shorter cold seasons. And,

this temperature increases is particularly predicted to increase by about 1–3°C by the mid and by about 2–5°C by the late twenty-first century. The report shows that at 2°C of global warming, heat extremes would more often reach critical tolerance thresholds for agriculture and health. Therefore, it is staminal that a lot of countries especially in the south of 40° north latitude including Turkey located in the geographic region where the adverse effects of climate change are possible struggle these climatic fluctuations, take action against anticipated threats, and revive strategies in this direction (Budak 2022). It have predicted to climatological extremes are caused various abiotic stresses and have a general negative effect on plant growth and development as also likely all living. Thus, future agricultural crop production and thus global food security will encounter additional challenges with human population increase competing for environmental resources (Bita and Gerats 2013; Pereira 2016). In this respect, performing genome-wide analyzes of stress-resistant genotypes from agricultural crop, revealing their tolerance and selective mechanisms to against adversely conditions, and cultivating new varieties are of great importance to tackle all these challenges.

Barley (*Hordeum vulgare* L.) is one of the most significant cereal crops farmed in Europe, the Middle East, North and South Africa, and Asia. This cereal, great economic value due to its use in both animal feed and the food industry are a cereal required grown in abiotic stress conditions that limit plant growth due to global climate changes in our current period. Furthermore, it is preferred as a model plant in cytogenetic researches for reasons such as its effortless supply, in vitro germination of seeds and small genome (Tabur and Demir 2010b; Özmen et al. 2022, 2023). Cytogenetic researches play an important role in understanding the chromosomal and genetic architecture of plant species. In particular, the chromosomal aberrations (CAs) have been accepted as an indicator of genetic damages and for those alterations which ultimately lead to mutations (Saxena 2022). Therefore, performing the CAs test has vital significant to determine whether a test substance or abiotic stress factors can cause various types of mutations over time.

Among the abiotic stresses, temperature increase has major negative impact on agricultural crops susceptible to changes in temperature. Temperature stress occur result of the cumulative effect of the temperature severity, the time the plant is exposed to these unfavorable condition, and the degree at which the temperature is increasing and cause significant and irreversible damage to plant growth and development (Hill and Li 2022). High temperatures are absolute effective as a stress factor in plants during germination, and the measures taken by plants and their molecular responses under these stress conditions are completely different. Each plant species has a temperature range represented by a minimum, maximum, and optimum in which it functions optimally, and outside this range all cellular metabolisms and thus plant growth are adversely affected (Hatfield and Prueger 2015). High temperature stress (HTS) disrupts the vital cellular phenomena by damaging generally physiological, biochemical and molecular mechanisms in plants (Narayanan 2018; Jacott and Boden 2020) and production of toxic metabolites and reactive oxygen species (ROS) takes place in the injured cells occurred as a result of aberrant metabolism (Wahid et al. 2007). Hence this situation causes total crop failure by decreasing in growth, product and quality (Shrestha et al. 2022; Khan et al. 2023). Increasing temperature inhibits different stages of plant development especially seriously reduces the germination and early seedling growth in a number of plant species including barley (Wahid et al. 2010). Additionally, it has been reported that HTS negatively affects cell division and microtubule organization in tobacco, wheat and vetch thus leading to decreased mitotic index (MI) and irregular mitotic configurations (Abou-Deif and Mohamed 2007; Öney and Tabur 2013; Öney et al. 2015). Fareghi et al. (2015) asserted that *Vicia dasycarpa* that are normally diploid exhibit a mixoploid state with diploid, aneuploid and tetraploid cells after temperature shock (boiling the seeds at 90°C for 3 min.). However, the ability of a genotype to survive at high temperature depends on the type or variety of the plant, age, stage of development, the susceptibility of the cell types, the degree and duration of the elevated temperature (Wahid et al. 2007; Hasanuzzaman et al. 2013).

Plant hormones are essential for regulating the interactions between plants and their complex biotic and abiotic environments. Most of the physiological activities occurring in the plant are under the control of these hormones. The effects of hormones always appear in a balance as complementary to each other (synergistic) or reducing the effect of each other (antagonistic) (Aerts et al. 2021). Under single or multifactorial stress combination phenomenon, fluctuations in hormonal balance in plants bring about serious morphological, physiological, biochemical and molecular changes (Goharrizi et al. 2021; Zandalinas et al. 2022 and their cited). For example, ABA (abscisic acid) plays a major role in different stages of plant development such as stomata opening and closing, seed germination, and dormancy and triggers many physiological mechanisms in plants. The plant growth is severely retarded and it increases the ABA concentration in cells under drought conditions. ABA

accumulation during this period controls transpiration and inhibit stomatal disclosure (Dong et al. 2018).

There are many studies that phytohormones or various PGR agents have positive effects on plant growth and development, as well as various physiological and biochemical mechanisms, and increase plant resistance against many stresses. Because the role of different individual phytohormones under abiotic stresses is too board to be covered here, we can direct readers to up-to-date research articles and reviews on the subject (Huyluoğlu et al., 2008; Moumita et al. 2019; Younis and Ismail 2019; Emamverdian et al. 2020; Islam and Mohammad 2020; Mangena 2020, 2022; Kosakivska et al. 2022; Kothari and Lachowiec 2021; Sharma 2021; El-Beltagi et al. 2022; Fatma et al. 2022; Sarwar et al. 2022; Shao et al. 2022; Verma et al. 2022; Sultan et al. 2023). The constantly rising ambient temperature caused by rapidly changing climate warming is considered one of the most detrimental abiotic stresses and heat tolerance in plants can be achieved by exogenous application of various protectant substances (Rasheed et al. 2011; Qureshi et al. 2022). Therefore, since especially recent ten years, the exogenous application of protectant substances such as osmoprotectants, phytohormones, signal molecules, polyamines, trace elements and nutrients have studied by numberless researchers to alleviate the harmful effects of HTS on plant (Hasanuzzaman et al. 2012; Waraich et al. 2012; Öney and Tabur 2013; Zaki et al. 2014; Öney et al. 2015; Kaur et al. 2018; Taheri and Haghighi 2018; Chen et al. 2019; Wu and Yang 2019; Alcázar et al. 2020; Jing et al. 2020; Li N. et al. 2021, Li Y. et al. 2023; Sharma et al. 2022; Wang et al. 2022; Wu et al. 2022; Huang et al. 2023; Hudelson 2023; Mei et al. 2023).

As mentioned above, there are many studies that phytohormones have positive effects on plant growth, development, physiological processes, and yield and increase plant stress resistance against various stresses. On the other hand, the effects of various phytohormones or PGRs on mitotic activity and chromosomal behaviors under normal conditions (in a stress-free environment) have also investigated by many researchers since the 1970s (Powell et al. 1973; Oh and Clouse 1998; Hu et al. 2000; İsmailoğlu et al. 2004; Huyluoğlu et al. 2008; Kartal et al. 2009; Truta et al. 2011; El-Ghamrey et al. 2013; El-Ghamery and Mousa 2017; Tabur et al. 2019; Tütünoğlu et al. 2019). It is a well-known fact that the exogenous application of both natural and synthetic PGRs contributes to the increase in the relative number of embryonic cells. Therefore most of these researchers agree that the exogenous phytohormones promotes cell division and proliferation and activates DNA replication and protein synthesis, but causes chromosomal aberrations (CAs) by disrupting the mitotic balance. Furthermore, some of these researchers argue that PGRs are more effective on cell division at high concentrations, while others assert that they are more effective at low concentrations.

However, studies on PGRs effects on cell division, mitotic activity and chromosome behavior under various stress conditions (heavy metals, salinity and drought) are quite limited (Mansour and Kamel 2005; Tabur and Demir 2009; 2010a,b; Maraklı et al. 2014; Özmen et al. 2022). Moreover, a single study was found on how effective only EBR are on these parameters (MI and CAs), especially under heat stress conditions as a result of our detailed literature research (Pradhan and Gupta 2013). For this reason, the effects of ten different PGRs either alone or double and triple combinations, mentioned on MI and chromosome behaviors under HTS have been comprehensively revealed in this study for the first time. As a result, aims of this work are (1) to determine the effect of HTS on MI and CAs, (2) to determine the effects of exogenous application of various PGRs alone or in double, triple combinations on the mitotic activity and chromosome behaviors in barley root meristems under nonstress conditions, (3) to comparatively evaluate the effects of these PGRs on the mentioned parameters in barley root meristems under HTS and to fill the gap in the literature on this subject, (4) to clarify in detail to what extent all studied PGRs and their combinations can overcome HTS, whether they encourage cells to enter mitosis, and whether they cause any changes in the structure and behaviors of chromosomes.

MATERIALS AND METHODS

Preparation of the seeds and PGRs

The barley seeds (*Hordeum vulgare* cv. Bülbül 89) were kindly provided from Field Crops Research Institute, Ankara/Turkey. PGRs used in the experiments were obtained from Fluka and Sigma-Aldrich Firm. To forbid contamination before germination experiments, the barley seeds were surface sterilized by immersion in 1% (w/v) NaClO solution for 10 min, rinsed thoroughly five times with sterile distilled water and dried on filter papers at room temperature. Ten different PGRs were used in the study: GA_3 (gibberellic acid), KIN (kinetin), BA (benzyladenine), E (ethylene), EBR (24-epibrassinolide), TRIA (triacontanol), Spm (spermine), Spd (spermidine), Put (putrescine) and Cad (cadaverine). The concentration of each PGR (as μM, micromolar), which reduces the damaging effect of HTS (30°C) on germination, was determined as a result of a preliminary study (Figure 1).

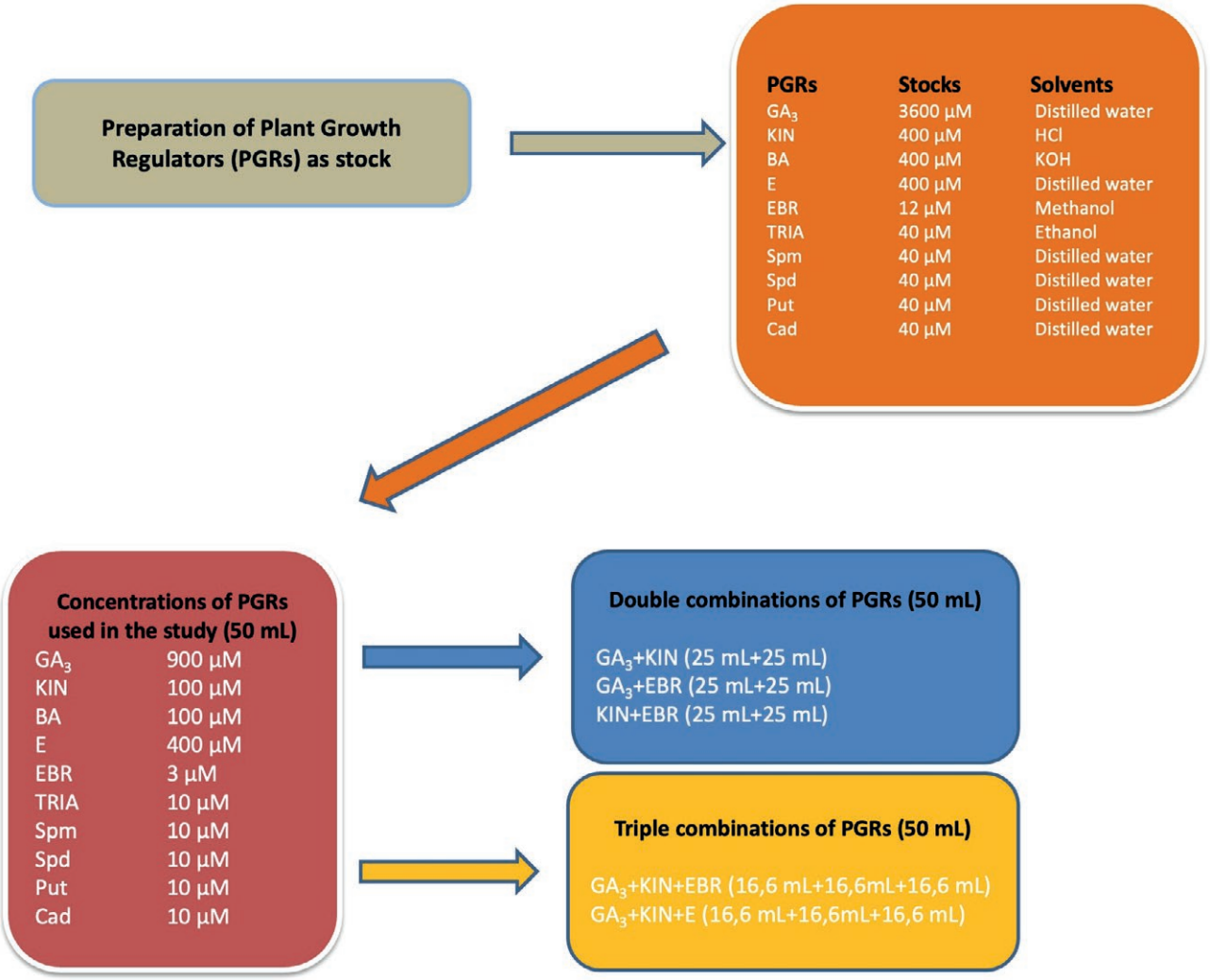

Figure 1. Diagram showing PGRs solvents, prepared stocks and concentration of solutions used in the study. Stock solutions were prepared by dissolving each of PGRs with appropriate solvents and made up to liter (µM, micromolar) with distilled water. The stock solutions were diluted and the concentrations of solutions used in the study were obtained. For this, PGR concentrations which reduce the damaging effect of 30°C, the tolerance limit of barley seeds against heat stress were used. Seeds were pretreated in 50 mL distilled water (control), PGRs alone and their double-triple combinations for 24 hours at room temperature. Germination process carried out at constant temperatures of 20°C (control) and 30°C in an incubator.

Germination experiments

First of all, germination experiments were carried out at different temperatures between 22 and 35°C and the tolerance limit of barley seeds against heat stress was determined as 30°C. Germination processes were carried out at constant temperatures of 20°C (control) and 30°C in the dark and in an incubator. For this process, full-looking, robust and uniform sized 20 seeds were selected first. These previously sterilized seeds were pretreated in 50 mL distilled water (control), GA_3, KIN, BA, E, EBR, TRIA, Spm, Spd, Put and Cad alone and in their double-triple combinations for 24 hours at room temperature. At the end of this pretreatment session, the solutions were filtered and the seeds were vacuum-dried. Then, the seeds for each application were arranged in Petri dishes covered with two sheets of filter paper moistened with 7 ml of distilled water. Immediately after sowing, the Petri dishes were placed in the above-mentioned 20°C and 30°C constant temperature incubators for germination. At the specified temperatures, they could not be studied because suitable and sufficient germination did not occur in combinations other than double combinations GA_3+KIN, GA_3+EBR, KIN+EBR and triple combinations GA_3+KIN +EBR, GA_3+KIN+E.

Cytogenetic examinations

For cytogenetic examinations, the root tips reached about 1 cm length after 5-7 day were excised, pretreated with a saturated solution of paradichlorobenzene for 4 h at 20 °C, fixed in solution absolute ethanol:glacial acetic acid (3:1, v/v) for overnight, and stored in 70% alcohol at refrigerator until used again. The root tips were hydrolyzed in 1 N HCl at 60 °C for 15–18 min, stained for 1-2 h in accordance with the standard procedure for Feulgen staining, smashed in a drop of 45% acetic acid and squashed (Elçi and Sancak 2013). After 24 h, microscopic slides were made permanent by mounting with alcohol vapor exchange method. The best mitotic phases and mitotic aberrances were photographed (100X) with digital camera (Olympus C-5060) mounted on an Olympus CX41 microscope.

Data analyses and statistical evaluations

For detect the effect of PGRs and HTS on the MI, the prepared slides were examined under the microscope at 100X magnification, and MI, i.e. percentage of dividing cells were accounted by counting at least 6000 cells for per application (three repeat, 2000 per slide). The MI was calculated using the following equation:

$$MI\ (\%) = \frac{\text{total number of dividing cells}}{\text{total number of analyzed cells}} \times 100$$

At the same time, CAs occurring at all stages of mitosis during microscopic observation of the slides were calculated according to the following the equation for each per-application as the percentage of 350 dividing cells counted.

$$CA\ (\%) = \frac{\text{total number of aberrant cells}}{\text{total number of dividing cells}} \times 100$$

All experiments were repeated three times. Statistical evaluation concerning all obtained parameters was realized by using SPSS 14.0 program according to Duncan's multiple range test, at $p \leq 0.05$ level of significance (Duncan 1955).

RESULTS

As explained in detail in the Introduction, it is known that under normal conditions, GA_3, cytokinins (CKs= BA and KIN) and E generally promote cell division and cell elongation, thereby increasing growth. However, the effects of EBR, TRIA and PAs (Spm, Spd, Put and Cad) on cell division have not yet been fully elucidated. Therefore, the present study aimed to clarify the counterchecks of these chemicals alone or in combination on MI and CAs, under both optimum (20˚C) and HTS (30˚C).

Counterchecks of PGRs against the adverse effects of HTS on MI

The MI values calculated as a result of cell counting of barley meristems after pre-application of various PGRs alone or in combinations at 20°C and 30°C are presented in Figure 2.

At optimum temperature (20°C), the application of GA_3, BA and Put, respectively, from the PGRs studied here showed statistically quite a lot successful effect on the MI of barley root meristems compared to the control group. Especially, at GA_3 the MI were reached to the highest value by increasing from 6,2±0,3 (at 20°C, in distilled water) to 15,1±0,8 (approx. 2.5 fold). While E, Spm and Cad applications were partially successful on this parameter KIN, EBR, TRIA and Spd applications exhibited an inhibitory effect on the MI. Considering the alone PGRs pre-applications, GA_3 treatment on the MI was more successful than all other treatments, while TRIA had the most negative effect. Among the double combinations of PGRs, GA_3+KIN showed a more successful effect on the MI than the control group and other double combinations. In addition, both of the triple combinations studied (GA_3+KIN+EBR and GA_3+KIN+E) displayed an excellent performance by showing a very successful effect on the MI compared to all other treatments except GA_3 alone. Considering all the PGRs applications both alone and in double/triple combinations, the most positive effect on the MI was obtained with the application of GA_3 alone and GA_3+KIN+E from the triple combinations. But, at the KIN+EBR, one of the double-combinations, the MI were recorded as the lowest value by decreasing from 6,2±0,3 to 1,5±0,5 (Figure 2).

At HTS (30°C), the MI in barley root meristem cells germinated in distilled water medium decreased by 35% compared to the control (at 20°C). When applied alone, it was determined that the PGRs, which showed a very successful performance on the MI of meristem cells at HTS compared to own control group (in distilled water/at 30°C), were GA_3, BA, E, Put and Spm, respectively. However, KIN, EBR, TRIA, Spd and Cad alone were not successful in alleviating the negative effect of HTS on the MI. Considering the pre-applications of all PGRs alone, GA_3 treatment increased from 5,7±0,3 (at 30°C, in distilled water) to 15,3±0,7 was more successful than the others in

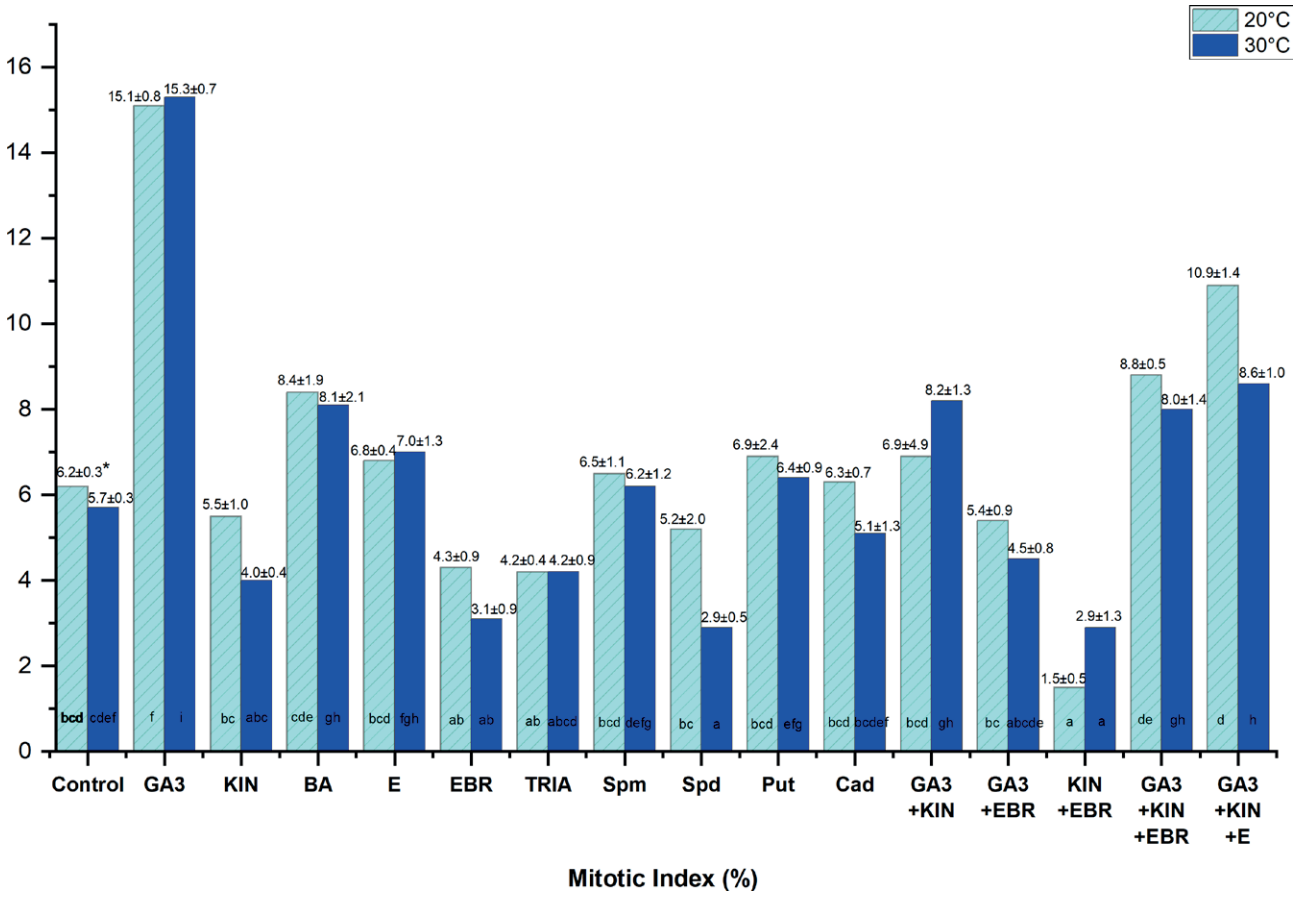

Figure 2. Mitotic index scores in meristem cells of barley exposed to high temperature stress after plant growth regulators supplementation.*Values with insignificant difference ($P \leq 0.05$) for each column are indicated with same letters (± Standard deviation). Seeds were germinated at constant temperatures of 20°C (control) and 30°C in the dark and in an incubator. As test solution, 900 µM GA_3, 100 µM KIN, 100 µM BA, 400 µM E, 3µM EBR, 10 µM TRIA and 10 µM PAs (Spm, Spd, Put and Cad) were used. The pretreatment process of seeds was performed by soaking 24 h in constant volumes (50 mL) of distilled water (control) or each PGR. All data were evaluated as three replicates.

mitigating the negative effect of HTS on the MI, but Spd was extremely unsuccessful on this parameter. Among the double combinations of PGRs, again GA_3+KIN was quite a lot successful in mitigating the negative effect of HTS on the MI compared to its control group and other double combinations. However, the KIN+EBR double combination had the lowest MI value together with Spd from the single combinations. Similarly, both of the triple combinations studied resulted in a statistically significant increase in the MI under HTS (Figure 2).

Among all studied applications, GA_3, E, GA_3+KIN and KIN+EBR showed a more positive effect on the MI compared to those at optimum temperature in barley meristem cells exposed to HTS. None of the other applications under HTS could reach MI values under own self optimum conditions. Under these conditions, double combinations (except GA_3+EBR) were more successful than single PGRs. Especially, while KIN+EBR had the lowest MI value (1,5±0,5) under optimum conditions, this value increased approximately twice and reached 2,9±1,3 at HTS (Figure 2).

Counterchecks of PGRs against the adverse effects of HTS on CAs

The percentages of CAs into barley meristem cells germinated in distilled water and at 20°C (control) and 30°C after pretreatment of various PGRs alone or in double/triple combinations were summarized in Figure 3. Representative images of CAs for all applications were given in Figure 4. As a result of cytological examinations, no aberration was found in the chromosome structures of barley meristem cells germinated in

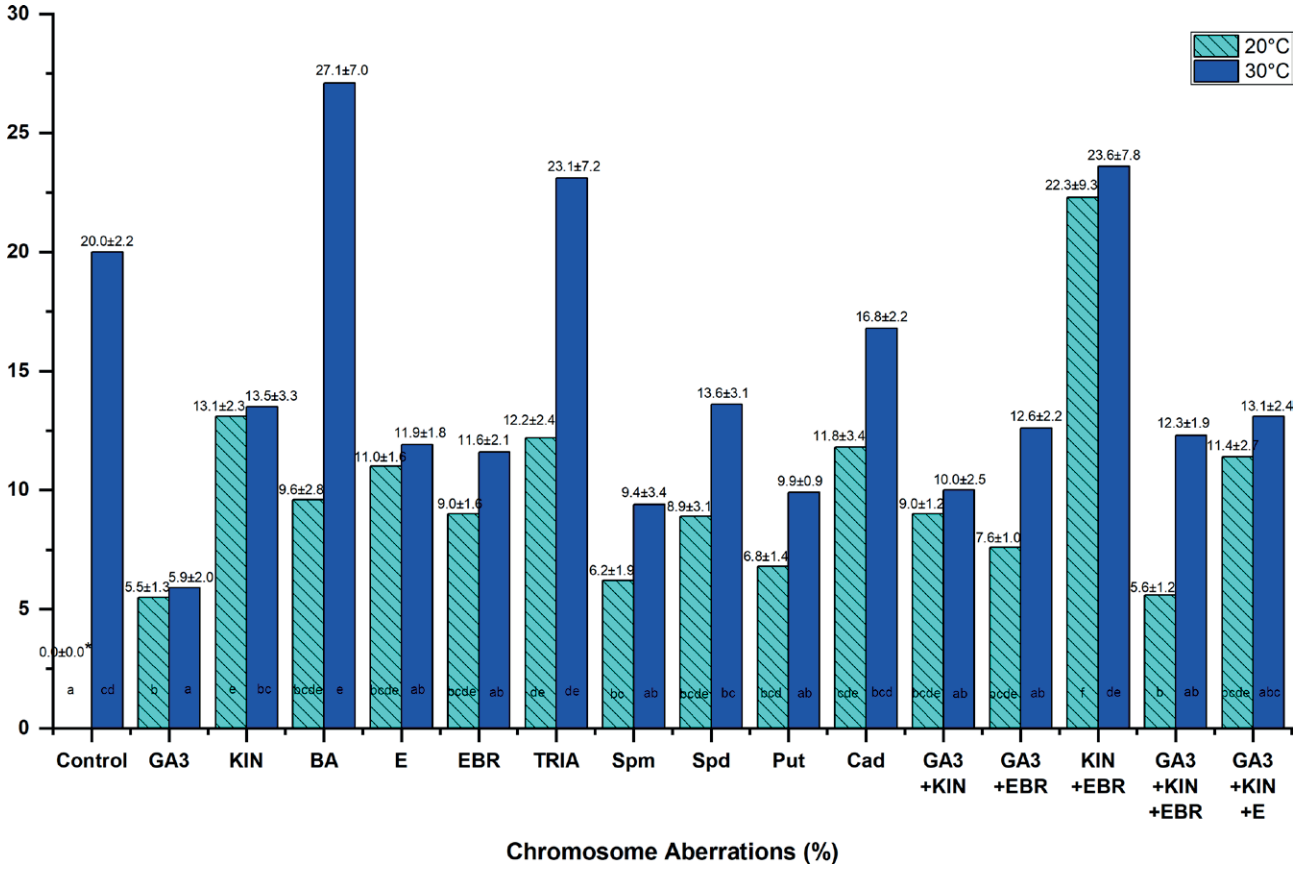

Figure 3. Frequency of chromosome aberrations in meristem cells of barley exposed to high temperature stress after plant growth regulators supplementation. *Values with insignificant difference (P ≤ 0.05) for each column are indicated with same letters (± Standard deviation). Seeds were germinated at constant temperatures of 20°C (control) and 30°C in the dark and in an incubator. As test solution, 900 µM GA_3, 100 µM KIN, 100 µM BA, 400 µM E, 3µM EBR, 10 µM TRIA and 10 µM PAs (Spm, Spd, Put and Cad) were used. The pretreatment process of seeds was performed by soaking 24 h in constant volumes (50 mL) of distilled water (control) or each PGR. All data were evaluated as three replicates.

distilled water and at 20°C and all of the mitotic stages were observed normally (Fig. 4 A-D). Whereas, the rate of CA in samples subjected to HTS (30°C) was determined to be 20,0±2,2%. Also, in terms of genotoxicity, these aberrations were observed to increase significantly (p≤0.05) with all PGR treatments compared to the control, as evidenced by the CA percentages. Under optimum conditions, the highest CA frequency was detected in KIN+EBR, one of the double combinations by increasing from 0,00 ±0,0% to 22,3±9,3%, followed by KIN (13,2±2,3%), TRIA (12,2±2,4%), Cad (11,8±3,4%) and GA_3+KIN+E (11,4±2,7%). The applications with the lowest CA frequency are also GA_3 alone (5,5±1,3%) and triple combination GA_3+KIN+EBR (5,6±1,2%) (Figure 3).

Under HTS, most of the PGR pretreatments studied greatly attenuated the negative effect on CA percentages in barley meristem cells. At the temperature level in mentioned, however, the percentage of CAs respectively, at BA (27,1±7,0%), KIN+EBR (23,6±7,8%) and TRIA (23,1±7,2%), increased even more compared to own control group (20,0±2,2%). It was determined that the GA_3 application alone was the most successful application (almost the same as optimum conditions) compared to all other combinations studied by reducing the detrimental effect of HTS on the percentage of CA from 20,0±2,2% to 5,9±2,0% (Figure 3).

In all PGR applications studied, HTS significantly increased CAs compared to own optimum conditions. Especially with BA, GA_3+KIN+EBR and TRIA applications respectively, CA rates increased by 2 times or more compared to own optimum conditions in HTS.

Microscopic images of a wide range of CAs observed in the preparations prepared with root tips belonging to all application groups are shown in Figure 4. Generally, the most extensive aberrations observed in all application were micronucleus (Figure 4 a, b), disorderly pro-

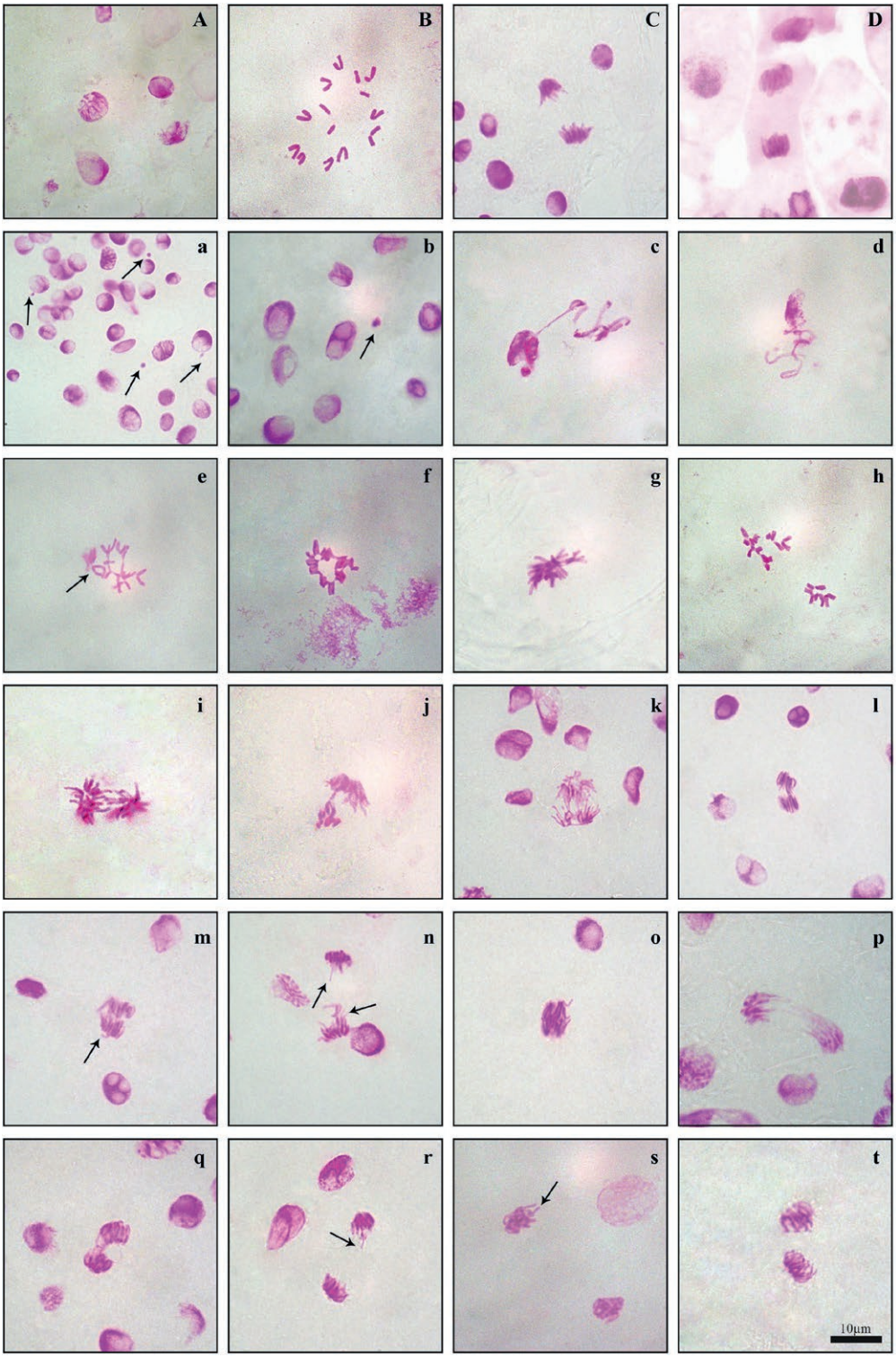

Figure 4. Representative images of microphotographs of normal mitotic stages **(A-D)** and aberrations **(a-t)** observed in all application groups studied in barley plant. **A** prophase, **B** metaphase (2n=14), **C** anaphase, **D** telophase. **a, b** micronucleus (arrows) **c** disorderly prophase with micronucleus **d** uncoiled chromosomes **e** chromosomal ringing (arrow) **f** vacuolated sequencing at metaphase **g** sticky chromosomes **h** chromosomal irregularity in the equatorial plane **i** stellar anaphase **j** disorderly anaphase **k, l** anaphase with multiple bridges **m** alignment anaphase with vagrant chromosome (arrow) **n** laggards in anaphase (arrows) **o** multipolar anaphase **p** polar slip in anaphase **q** bridges in telophase **r** laggard in telophase (arrow) **s** vagrant chromosome in telophase (arrow) **t** polar slip in telophase. Scale bar = 10 µm.

anaphase (Figure 4 c, j), uncoiled chromosomes (Figure 4 d), sticky chromosomes (Figure 4 g), chromosomal irregularity in the equatorial plane (Figure 4 h), alignment anaphase (Figure 4 m), multipolar anaphase (Figure 4 o), laggard and vagrant chromosomes (Figure 4 m, n, r, s), bridges (Figure 4 k, l, q) and polar slip (Figure 4 p, t) in ana-telophase. The minimal level aberrations recorded were chromosome ringing (Figure 4 e), vacuolated sequencing at metaphase (Figure 4 f) and stellar anaphase (Figure 4 i).

DISCUSSION

As a result of the extensive literature review, adequate study were not found on the effects of some of the PGRs studied here (especially EBR, TRIA and PAs) on cell division and chromosomal behaviors. Moreover, it was seen that there is still no consensus among researchers about the role of also GA_3, KIN, BA and E on these parameters. For this reason, it was found appropriate to compare the effects of these parameters under optimum conditions before moving on to the effects of the above mentioned PGRs under HTS conditions.

Effects of exogenous PGRs on cytotoxicity and genotoxicity at optimum conditions

In this part of the study, the effects cytotoxic and genotoxic the effects of hormone/with hormone-like activity shown chemicals such as exogenously GA_3, KIN, BA, E, EBR, TRIA and PAs in the barley meristem cells of at optimum conditions were investigated. The results were compared with the relevant literature and among themselves.

According to our findings, while the MI value of barley seeds in the control group (in distilled water, at 20°C) was 6,2±0,3%; KIN, EBR, TRIA, Spd, GA_3+EBR and KIN+EBR treatments could not reach this (see Figure 2). Similarly, some researchers suggested that externally applied GA_3, KIN, BA (Tabur and Demir 2010a; Tütünoğlu et al. 2019) and TRIA application (Tabur and Demir 2008a) under stress-free conditions reduced mitotic activity in barley root meristems. In that case, it can be said that exogenously application of some stimulatory growth regulators under normal conditions without stress may be useless. On the other hand, it has been reported that exogenous GA_3 (Mansour and Kamel 2005; MacDonald and Little 2006), low concentration BA (Huyluoğlu et al. 2008; Truta et al. 2011; El-Ghamrey et al. 2013) and TRIA (Hangarter and Ries 1978) applications promote cell division and thus MI during germination under normal conditions. However, the effects of E, BRs and PAs on cell division have not been fully elucidated. It has been stated that these PGRs may have positive or negative effects on cell division. Some researchers asserted that these PGRs promote cell division and MI at low concentrations (Kartal et al. 2009; Maraklı et al., 2014), while at high concentrations reported that they had an inhibitory effect (Hu et al., 2000; İsmailoğlu et al., 2004; Tabur and Demir, 2009, 2010 a,b; Özmen et al. 2022). Our findings reveal that the application of GA_3, BA and Put alone had statistically a very successful effect on the MI of barley seeds compared to the control group and also E, Spm and Cad applications were had partially successful. But, TRIA, EBR, Spd and KIN applications had an inhibitory effect on this parameter (see Figure 2). Considering the PGRs studied, either alone or in double-triple combinations, it is seen that the most positive effect on the MI is obtained with GA_3 pre-application alone (15,1±0,8%) and generally the combinations with GA_3 are statistically more significant than the control group (6,2±0,3%). For example, the GA_3+KIN+EBR triple combination created with the addition of GA_3 to the KIN+EBR double application, where the most negative effect on the MI was observed, increased statistically significantly the MI compared to the control group. Moreover, considering all the PGR applications studied, the most positive effect on the MI was obtained with the application of GA_3 alone and GA_3+KIN+E from the triple combinations (see Figure 2). This indicates that GA_3 has an indispensable place in cell division. Data on double and triple combinations of PGRs studied here on MI under optimum conditions are presented for the first time in this study.

No any chromosomal abnormalities (CA) were came across in barley root meristems germinated under optimum conditions. However, as a result of the PGRs pretreatment studied here, either alone or in double/triple combinations, various types and percentages of CAs were generally observed (see Figure 3-4). This is due to the fact that even any externally applied stimulator under optimum conditions is perceived as a stress factor by the plant. The least percentage of CA was obtained with GA_3 pretreatment alone. In particular, it was determined that the percentage of CA in seeds with KIN pretreatment was higher than other PGRs applied alone. Among all the combinations studied, the most CA again was observed in the KIN+EBR application, which is one of the double combinations by increasing from 0,00±0,0% abnormal cells (at distilled water, control) to 22,3±9,3%. Moreover, the CA ratio was reduced in the GA_3+KIN+EBR triple combination (5,6±1,2%) formed by adding GA_3 to this double combination until to the

level in the GA_3 application alone (5,5±1,3%) (see Figure 3). In this case, as mentioned above, we can say that the negative effect of KIN+EBR double application on the MI also is due to these CAs caused by the mitotic irregularities during cell division. Again, data on double and triple combinations of PGRs studied under optimum conditions on CAs also are presented for the first time in this study.

In our study, it was observed that CKs and E caused the formation of CAs such as micronucleus, disorderly pro-anaphase, chromosome ringing, chromosomal irregularity in the equatorial plane, multipolar anaphase, sticky and uncoiled chromosomes, especially bridges in ana-telophase. In addition, CAs such as sticky chromosomes, chromosome ringing, laggard and vagrant chromosomes in ana-telophase, and alignment anaphase were frequently encountered in GA_3, EBR and TRIA applications. On the other hand, it has been determined that PAs cause CAs in the form of sticky chromosomes, disorderly anaphase, chromosome bridges in ana-telophase, and polar slip in ana-telophase (see Figure 4 a-t).

Information on the effects of various PGRs on chromosome behavior under optimum conditions is limited to only a few studies conducted in the last 20 years. It has been reported that high concentrations of CKs negatively affect chromosomal behaviors with a clastogenic effect and cause different types of genetic and chromosomal variations (Huyluoğlu et al. 2008; Truta et al. 2011; El-Ghamrey et al. 2013; El-Ghamery and Mousa 2017). However, Tabur and Demir (2010a) reported in their study that BA and GA_3 application did not cause any chromosomal abnormality, but KIN and E application increased CAs significantly compared to the control. According to Tütünoğlu et al. (2019) argue that increasing GA_3 concentrations depending on time and dose show cytotoxic and genotoxic effects and the difference between control and treatment groups is statistically significant, while Mansour and Kamel (2005) argue that there is statistically an insignificant increase in CAs. Again, some researchers reported that exogenous applied TRIA, EBR and HBRs under optimum conditions negatively affect chromosomal behavior in barley meristems (Tabur and Demir, 2008a, 2009; Kartal et al., 2009). Similarly, Ünal et al. (2002) on barley seeds, İsmailoğlu et al. (2004) on diploid, tetraploid and hexaploid wheat seeds in their studies stated that also PAs cause mitotic irregularities. Tabur and Demir (2010b) reported that PAs inhibited the MI in barley meristems, significantly increased the CAs of other PAs except Spd and Put had the highest abnormality rate in total. Özmen et al. (2022) also stated that PAs significantly increased the CA rate by causing various mitotic abnormalities, and the PA with the highest abnormality percentage was Spm. If a comparison is made in the light of all these studies; it can be said that the effects of PGRs on MI and CAs under optimum conditions may differ depending on the plant species studied, plant development stages, genotype, used concentration, exposure time and pre-application method.

Effects of exogenous PGRs on cytotoxicity and genotoxicity at HTS conditions

HTS may inhibit seed germination and mitosis, thereby reducing germination rate (Çavuşoğlu and Kabar, 2007; Sharma et al. 2022) and mitotic activity (Öney and Tabur, 2013; Öney et al. 2015). It may be cause a decrease in the amount of protein and stop the synthesis of proteins that act as osmoprotectants that play a role in temperature tolerance (Xu et al. 2021). According to the results obtained from our study, it has been confirmed once again that HTS reduces the MI also in barley plant, and limited literature information on this subject has been contributed. The reason for the decrease in mitotic activity at high temperature may be directly or may be related to the loss of enzyme activation, which is responsible for mitosis, and also proteins denaturation and lipid peroxidation (Sheikhi et al. 2023).

At the same time, HTS showed quite unfavorable effects on the chromosome behavior of barley seeds. In our study, it was determined very high rate and various types of chromosome aberrations in barley root meristems germinated at 30°C (see Figure 3-4). These aberrations may be due to the damaging effects of HTS on microtubule organization (Wahid et al. 2007), which may have led to irregular mitotic configurations and CAs, mainly involving spindle fibers and metaphase (Abou-Deif and Mohamed, 2007). In addition, it has been reported that reactive oxygen species (ROS) such as hydroxyl (OH), superoxide (O_2-), hydrogen peroxide (H_2O_2) and single oxygen ($^1O_2$), which occur due to HTS may be cause deaggregation of DNA, RNA and nucleic acids (Liu and Huang, 2000). Faraghi et al. (2015) suggest that *Vicia dasycarpa*, which is normally diploid, exhibits a mixoploid state with aneuploid and tetraploid cells after temperature shock. However, Öney and Tabur (2013) reported in their study that high temperature (30°C) did not cause any CA in *Vicia faba* root meristem cells. In the light of all these studies, we can emphasize again that heat stress may have different effects depending on the type of plant used, the severity and duration of the application of stress. Because the upper and lower threshold limits of abiotic stresses can show different effects in different species, sometimes even in different

varieties of the same species (Wahid et al. 2007; Hemantaranjan et al. 2014).

On the other hand, it was determined that PGRs were effective at different degrees in mitigating the negative effects of HTS on the MI, and this difference was statistically significant (see Figure 2). Especially KIN, EBR, TRIA, Spd, Cad, GA_3+EBR and KIN+EBR applications have not been successful in alleviating the negative effects of HTS on the MI. It was observed that the most negative effect on the MI occurred with Spd application alone and KIN+EBR application from double combinations. In this case, it would not be right to expect every stimulator to be successful in overcoming the heat stress on this parameter. Indeed, it has been emphasized by many researchers that the type(s) and concentrations of stimulators may vary from species to species in overcoming various abiotic stresses (Mirza and Bagni 1991; Tabur and Demir 2010a,b; Korek and Marzec 2023). However, considering all PGR pre-applications, either alone or in double/triple combinations, in our study, more than half of the tested applications showed a successful performance in alleviating the unfavorable effect of HTS on the MI. In particular, the most positive effect was obtained with the application of GA_3 alone, GA_3+KIN from double combinations, and GA_3+KIN+E from triple combinations (see Figure 2). In addition, in the case of HTS alone GA_3 and GA_3+KIN double combinations reached a higher value than they have shown success under optimum conditions. Moreover, considering the success of other combinations with GA_3 on the MI, it is seen that again GA_3 has an indispensable place in alleviating the negative effects of stress compared to their own control groups (at 30°C in distilled water). Similarly, GA_3+KIN (8,2±1,3%) combination was more successful than KIN alone (4,0±0,4%) and GA_3+EBR (4,5±0,8%) combination was more successful than EBR alone (3,1±0,9%) in overcoming HTS on the MI. Also, the excellent success of the GA_3+KIN+EBR (8,0±1,4%) triple combination on the MI compared to the KIN+EBR (2,9±1,3%) double combination indicates that GA_3 creates a noticeable synergism with these PGRs. It has also been emphasized in previous reports that combinations with GA_3 against abiotic stresses are more effective role on seed germination and MI (Çavuşoğlu and Kabar 2007; Tabur and Demir 2008b). With this together, considering that the internal amount of stimulators such as CKs (El-Mashad and Kamel, 2001) and GAs (Prakash and Prathapasenan, 1990) decreases in seeds under stress conditions, these externally applied promoters is not surprising that they increase mitotic activity; it can be expect.

Although the information about the response of PGRs to stress factors during cell division has not been sufficiently clarified, it is known that various priming applications increase resistance to stress factors by promoting cell division, DNA replication (Giri and Schillinger 2003) and antioxidative defense (Afzal et al. 2006). The most common response under stress conditions is the acceleration of synthesis of protective components, especially osmoprotectants. Based on general literature information, it would be correct to say that the PGRs may have been successful in alleviating the damaging effect of HTS on the MI by increasing the activity of enzymes involved in cell division or by accelerating the synthesis of proteins that act as osmoprotectants that play a role in temperature tolerance. However, as mentioned in the Introduction, there is only one study (Pradhan and Gupta 2013) on the effects of these PGRs on mitotic activity, especially under high temperature conditions. In this previous study, it was reported that only EBR application was studied and increased the MI in *Brassica oleracea* var. *botrytis* root meristems germinated under low (4°C) and high (44°C) temperature stress. Contrary to our findings, these researchers suggested that increasing concentrations of EBR under high and low temperature stress increased MI. This paradox may be due to the type of plant, the concentration of EBR used and the applied temperature degree.

As for CAs, so far no studies have been conducted on the effects of all the above-mentioned PGRs on this parameter under HTS. Therefore, our study includes the first findings describing the data obtained on this parameter in detail. Accordingly, it was determined that the studied PGRs also showed statistically significant effects on the percentages of CAs in barley seeds germinated under HTS (see Figure 3). Although most of the PGRs applied alone or in double/triple combinations were successful in improving the CAs caused by HTS, only BA, TRIA and KIN+EBR applications could not show sufficient success on this parameter. Especially among all applications, the most positive effect on CAs was obtained again with the GA_3 application alone, while the most damaging effect was in BA application. Thus, the GA_3 application demonstrated once again on CAs its successful performance on the MI under HTS. For example; GA_3+KIN double application (10,0±2,5%) was more successful than KIN application alone (13,5±3,3%) in ameliorating the damaging effects of HTS by reducing the percentage of CAs.

Various mitotic aberrations were observed during microscopic scans of root meristem cells of barley seeds belonging to all application groups (see Figure 4 a-t). Aneugenic and clastogenic impacts that form an important portion of CAs might have been largely resulted from spindle dysfunction and chromosomal breaks respectively. The CAs, such as bridges and break, are

indicators of a clastogenic action, whereas chromosome losses, laggards, sticky, multipolarity and C-metaphase originate from aneugenic effects (Silveira et al. 2017). As known, accurate chromosome segregation in mitosis requires that sister kinetochores attach to microtubules emanating from opposite spindle poles (biorientation). Because kinetochore attachment is a stochastic process, it is error prone and can result in chromosome malorientation (Banerjee et al. 2020). Mitodepressive actions such as disorderly pro-anaphase, alignment anaphase, multipolar anaphase, stellar anaphase, bridges and polar slip in ana-telophase may be mainly the result of the above reasons. Moreover, Tabur and Demir (2010b) asserted that the nucleoplasmic bridges in ana-telophase might have been occurs as a consequence of inversions while Bonciu et al. (2018) have asserted originate from dicentric chromosomes or occur as a result of as faulty longitudinal break of sister chromatids during anaphase. Fiskesjö (1997) have claimed also that bridges are clastogenic effects, both resulting from chromosome and chromatid breaks. The large micronucleus (MN) in the cell indicates aneugenic effect resulting from chromosome loss while small MN indicates clastogenic effect due to chromosome breaks (Kontek et al. 2007). Briand and Kapoor (1989) have reported that the MNs are likely the consequence of vagrant chromosomes and fragments. Uncoiled chromosomes and chromosome ringing's may be the result of a weak mitotic effect and irregular chromosome contractions (Tabur and Demir 2010b). Asita and Mokhobo (2013) asserted that sticky chromosomes could be originated from abnormal DNA condensation, irregular chromosomal wrapping and inactivation of the axes. At the same time, such aberrations may be a result of improper folding of the chromatin fibers (Klášterská et al. 1976). According to some researchers, sticky chromosomes are a marker of high toxic effect on chromatin and irreversibility of the change (Fiskesjö and Levan 1993; Türkoğlu 2007). Chromosomal irregularity in the equatorial plane and vacuolated sequencing at metaphase may originate from unequal distribution of chromosome and spindle dysfunction. Laggard and vagrant chromosomes occurs during the anaphase where one or more chromatids gets detached from the rest of the chromatids and is incapable of moving towards the poles. Aberrations of these kinds may have occurred due to a weak mitotic impresses a consequence of failures in chromosomal attachment to the mitotic spindle (Patil and Bhat 1992).

Generally, it was concluded that BA, E, Put and Spm, respectively, among the PGRs alone studied, including at first GA_3, showed a very successful performance statistically in mitigating the negative effect of HTS on the MI. In addition, when the effects of the double/triple combinations of these PGRs on this parameter were evaluated, it was determined that all the studied combinations, except the KIN+EBR application from the double combinations, showed a superior success in overcoming the negative effect of HTS on the MI. In fact, this success was higher than most of the PGRs applied alone (see Figure 2). On the other hand, as a result of the statistical evaluations, it was proved that all PGRs studied, except for KIN+EBR, BA and TRIA applications, both alone and in double/triple combinations, showed an important successfully in the improvement of CAs (see Figure 3).

CONCLUSION

Various growth agents can be effective in different events in different species, even in individuals of the same species, and can be found in different amounts. Accordingly, which hormone is in effective concentration in any event in a plant this hormone would be responsible for growth and development events by performing its function. Indeed, as Khan (1971) points out, any event is unlikely to be governed by the absolute presence or absence of a hormone. In response to environmental conditions, some hormones in the plant may be more effective, some may be less effective or not effective at all. Therefore, it seems more plausible that whichever hormone is most effective, it functions in the relevant case.

In our study, the interactions between mitotic activity and mitotic irregularities and various stimulating growth agents, which can be counted as possible mechanisms of tolerance to increased heat stress as a result of global climate changes, were examined in barley, an important cereal crop. Thus, it has been tried to serve to fill a gap in the literature regarding these parameters. It is thought that the use of suitable PGRs for plants that will be grown in regions exposed to high temperatures will provide very beneficial results economically. However, a detailed investigation of the effects of these chemicals on basic metabolic events such as hydrolase synthesis and activity, nucleic acid metabolism, protein and enzyme synthesis, which can be directly or indirectly effective on mitotic activity, will help to elucidate the mechanism in question.

Consequently, thanks to these and similar studies, it can be contribute to the development of genetically temperature-tolerant products by changing the plant's sensing, signaling and regulatory pathways without disturbing other vital processes. In addition, a comprehensive explanation of the response of plants to high temperature tolerance and temperature tolerance mechanisms

and the development of possible strategies in this regard are mandatory. Therefore, it is necessary to map gene loci related to thermotolerance and to elucidate different genetic approaches that provide tolerance to heat stress (Asthir, 2015).

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AUTHOR CONTRIBUTIONS

ST and ŞBYE designed and performed the experiments. SÖ helped to conduct the experiments. ST wrote the manuscript. All authors read and approved the manuscript and have equal contribution.

REFERENCES

Abou-Deif MH, Mohamed FI. 2007. Effect of heat stress on chromosomes and protein patterns in six hexaploid wheat varieties. Res J Cell Mol Biol. 1(1): 42-49.

Aerts N, Mendes MP, Van Wees SCM. 2021. Multiple levels of crosstalk in hormone networks regulating plant defense. Plant J. 105: 489-504.

Afzal I, Basara SMA, Faooq M, Nawaz A. 2006. Alleviation of salinity stress in spring wheat by hormonal priming with ABA, salicylic acid and ascorbic acid. Int J Agric Biol. 8: 23-28.

Alcázar R, Bueno M, Tiburcio AF. 2020. Polyamines: Small amines with large effects on plant abiotic stress tolerance. Cells (MDPI). 9: 2373.

Asita AO, Mokhobo MM. 2013. Clastogenic and cytotoxic effects of four pesticides used to control insect pests of stored products on root meristems of *Allium cepa*. Environ Nat Resour Res. 3(2): 133-145.

Banerjee A, Adames N, Peccoud J, Tyson JJ. 2020. A stochastic model for error correction of kinetochore-microtubule attachments in budding yeast. PLoSONE. 15(8): e0236293.

Bita CE, Gerats T. 2013. Plant tolerance to high temperature in a changing environment: scientific fundamentals and production of heat stress-tolerant crops. Front Plant Sci. 4: 273.

Bonciu E, Firbas P, Fontanetti CS, Wusheng J, Karaismailoğlu MC, Liu D, Menicucci F, Pesnya DS, Popescu A, Romanovsky AV, Schiff S, Ślusarczyk J, Souza CP, Srivastava A, Sutan A, Papini A. 2018. An evaluation for the standardization of the *Allium cepa* test as cytotoxicity and genotoxicity assay. Caryologia. 71(3): 191-209.

Briand CH, Kapoor BM.1989. The cytogenetic effects of sodium salicylate on the root meristem cells of *Allium sativum* L. Cytologia. 54: 203-209.

Budak Ü. 2022. Possible effects of global climate change on Turkey's plant diversity. In: Doğan H, Fidan H, editors. Global Climate Change: Agriculture and Food Science Perspective. Iksad Publishing House, Ankara- Turkey, pp. 149-165.

Chen D, Shao Q, Yin L, Younis A, Zheng B. 2019. Polyamine function in plants: Metabolism, regulation on development, and roles in abiotic stress responses. Front Plant Sci. 9: 1945.

Çavuşoğlu K, Kabar K. 2007. Comparative effects of some plant growth regulators on the germination of barley and radish seeds under high temperature stress. Eur-Asian J BioSci. 1: 1-10.

Dong H, Bai L, Chang J, Song CP. 2018. Chloroplast protein PLGG1 is involved in abscisic acid-regulated lateral root development and stomatal movement in *Arabidopsis*. Biochem Biophys Res Commun. 495: 280-285.

Duncan DB. 1955. Multiple range and multiple F tests. Biometrics. 11: 1-42.

El-Beltagi HS, Ismail SA, Ibrahim NM, ShehataWF, Alkhateeb AA, Ghazzawy HS, El-Mogy MM, Sayed EG. 2022. Unravelling the effect of triacontanol in combating drought stress by ımproving growth, productivity, and physiological performance in strawberry plants. Plants. 11: 1913.

Elçi Ş, Sancak C. 2013. SİTOGENETIKTE ARAŞTIRMA YÖNTEMLERI VE GÖZLEMLER [Research methods and observations in cytogenetics]. Ankara Univ Pub House. Beşevler/ANKARA, 227 p. Turkish.

El-Ghamrey AA, Mahgoub HAM, Mousa MA. 2013. Effect of plant growth regulators on protein banding profile of some higher plants in relation to mitotic activity and the total abnormalities. Int J Adv Res. 1(10): 980-993.

El-Ghamery AA, Mousa MA. 2017. Investigation on the effect of benzyladenine on the germination, radicle growth and meristematic cells of *Nigella sativa* L. and *Allium cepa* L. Ann Agric Sci. 62: 11–21.

El-Mashad AAA, Kamel EA. 2001. Amelioration of NaCl stress in *Pisum sativum*. Indian J Exp Biol. 39(5): 469-475.

Emamverdian A, Ding Y, Mokhberdoran F (2020) The role of salicylic acid and gibberellin signaling in plant responses to abiotic stress with an emphasis on heavy metals. Plant Signal Behav 15 :7, 1777372.

Fareghi S, Rashidi V, Haghighi AR. 2015. Investigating the effect of heat shock on chromosomal aberration of *Vicia dasycarpa*. Cumhuriyet Sci J. 36(3): 657-663.

Fatma M, Asgher M, Iqbal N, Rasheed F, Sehar Z, Sofo A, Khan NA. 2022. Ethylene signaling under stressful environments: analyzing collaborative knowledge. Plants. 11: 2211.

Fiskesjö G. 1997. *Allium* test for screening chemicals; evaluation of cytological parameters. In: Wang W, Lower WR, Gorsuch JW, Hughes JS (eds) Plant for Environmental Studies. Boca Raton, New York: CRC Lewis Publishers pp. 308-333.

Fiskesjö G, Levan A. 1993. Evaluation of the first ten MEIC chemicals in the *Allium* test. Altern Lab Anim (ATLA). 21: 139-149.

Giri GS, Schillinger WF. 2003. Seed priming winter wheat for germination, emergence and yield. Crop Sci. 43(6): 2135-2141.

Goharrizi KJ, Hamblin MR, Karami S, Nazari M. 2021. Physiological, biochemical, and metabolic responses of abiotic plant stress: salinity and drought. Turk J Bot. 45: 623-642.

Hangarter R, Ries SK. 1978. Effect of triacontanol on plant cell cultures *in vitro*. Plant Physiol. 61: 855-857.

Hasanuzzaman M, Nahar K, Alam MM, Fujita M. 2012. Exogenous nitric oxide alleviates high temperature induced oxidative stress in wheat (*Triticum aestivum*) seedlings by modulating the antioxidant defense and glyoxalase system. Aust J Crop Sci. 6(8): 1314-1323.

Hasanuzzaman M, Nahar K, Alam MM, Roychowdhury R, Fujita M. 2013. Physiological, biochemical, and molecular mechanisms of heat stress tolerance in plants. Int J Mol Sci. 14: 9643-9684.

Hatfield JL, Prueger JH. 2015. Temperature extremes: Effect on plant growth and development. Weather Clim Extrem. 10: 4-10.

Hemantaranjan A, Bhanu AN, Singh MN, Yadav DK, Patel PK, Singh R, Katiyar D. 2014. Heat stress responses and thermotolerance. Adv Plants Agric Res. 1(3): 62-70.

Hill CB, Li C. 2022. Genetic improvement of heat stress tolerance in cereal crops. Agronomy. 12(5): 1205.

Hu Y, Bao F, Li J. 2000. Promotive effect of brassinosteroids on cell division involves a distinct CycD3-induction pathway in *Arabidopsis*. Plant J. 24(5): 693-701.

Huang J, Zhao X, Bürger M, Chory J, Wang X. 2023. The role of ethylene in plant temperature stress response. Trends Plant Sci. 28(7): 808-824.

Hudelson TJ. 2023. Elevated atmospheric ethylene and high temperature independently inhibit fruit set but not vegetative growth in tomato. Hortsci. 58(3): 247-253.

Huyluoğlu Z, Ünal M, Palavan-Ünsal N. 2008. Cytological evidences of the role of meta-topolin and benzyladenin in barley root tips. Adv Mol Biol. 1: 31-37.

IPCC 2021 Climate change widespread, rapid, and intensifying-IPCC. Available from: https://www.ipcc.ch/2021/08/09/ar6-wg1-20210809-pr/ [Accessed: May 05, 2023]

Islam S, Mohammad F. 2020. Triacontanol as a dynamic growth regulator for plants under diverse environmental conditions. Physiol Mol Biol Plants. 26(5): 871-883.

İsmailoğlu I, Ünal M, Palavan-Ünsal N. 2004. Effects of spermidine, spermine and cyclohexylamine on mitotic activity of 2X, 4X and 6X wheats. J Cell Mol Bio. 3: 83-88.

Jacott CN, Boden SA. 2020. Feeling the heat: developmental and molecular responses of wheat and barley to high ambient temperatures. J Exp Bot. 71 (19): 5740-5751.

Jing J, Guo S, Li Y, Li W. 2020. The alleviating effect of exogenous polyamines on heat stress susceptibility of different heat resistant wheat (*Triticum aestivum* L.) varieties. Sci Rep. 10: 7467.

Kartal G, Temel A, Arican E, Gözükırmızı N. 2009. Effects of brassinosteroids on barley root growth, antioxidant system and cell division. Plant Growth Regul. 58: 261-267.

Kaur H, Sirhindi G, Bhardwaj R, Alyemeni MN, Siddique KH, Ahmad P. 2018. 28-homobrassinolide regulates antioxidant enzyme activities and gene expression in response to salt-and temperature-induced oxidative stress in *Brassica juncea*. Sci Rep. 8: 8735.

Khan AA. 1971. Cytokinin: Permissive role in seed germination. Science. 171: 853-859.

Khan AH, Min L, Ma Y, Zeeshan M, Jin S, Zhang X. 2023. High-temperature stress in crops: male sterility, yield loss and potential remedy approaches. Plant Biotechnol J. 21: 680-697.

Klášterská I, Natarajan AT, Ramel C. 1976. An interpretation of the origin of subchromatid aberrations and chromosome stickiness as a category of chromatid aberrations. Hereditas. 83:153–162.

Kontek R, Osiecka R, Kontek B. 2007. Clastogenic and mitodepressive effects of the insecticide dichlorvos on root meristems of *Vicia faba*. J Appl Genet. 48(5): 359-361.

Korek M, Marzec M. 2023. Strigolactones and abscisic acid interactions affect plant development and response to abiotic stresses. BMC Plant Biol. 23: 314.

Kosakivska IV, Vedenicheva NP, Babenko LM, Voytenko LV, Romanenko KO, Vasyuk VA. 2022. Exogenous phytohormones in the regulation of growth and development of cereals under abiotic stresses. Mol Biol Rep. 49(1): 617-628.

Kothari A, Lachowiec J. 2021. Roles of brassinosteroids inmitigating heat stress damage in cereal crops. Int J Mol Sci. 22: 2706.

Li N, Euring D, Cha J., Lin Z, Lu M, Huang LJ, Kim WY. 2021. Plant hormone-mediated regulation of heat tolerance in response to global climate change. Front Plant Sci. 11: 627969.

Li Y, Han X, Ren H, Zhao B, Zhang J, Ren B, Gao H, Liu P. 2023. Exogenous SA or 6-BA maintains photosynthetic activity in maize leaves under high temperature stress. Crop J. 11(2): 605-617.

Liu X, Huang B. 2000. Heat stress injury in relation to membrane lipid peroxidation in creeping bent grass. Crop Sci. 40(2): 503-510.

MacDonald JE, Little CH. 2006. Foliar application of GA_3 during terminal long-shoot bud development stimulates shoot apical meristem activity in *Pinus sylvestris* seedling. Tree Physiol. 26(10): 1271-1276.

Mangena P. 2020. Role of benzyladenine seed priming on growth and physiological and biochemical response of soybean plants grown under high salinity stress condition. Int J Agron. 8847098.

Mangena P. 2022. Evolving role of synthetic cytokinin 6-benzyl adenine for drought stress tolerance in soybean (*Glycine max* L. Merr.). Front Sustain Food Syst. 6: 992581.

Mansour MM, Kamel EAR. 2005. Interactive effect of heavy metals and gibberellic acid on mitotic activity and some metabolic changes of *Vicia faba* L. plants. Cytologia. 70(3): 275-282.

Maraklı S, Temel A, Gözükırmızı N. 2014. Salt stress and homobrassinosteroid interactions during germination in barley roots. Not Bot Horti Agrobot Cluj-Nap. 42(2): 446-452.

Mei W, Chen W, Wang Y, Liu Z, Dong Y, Zhang G, Deng H, Liu X, Lu X, Wang F, Chen G, Tang W, Xiao Y. 2023. Exogenous kinetin modulates ROS homeostasis to affect heat tolerance in rice seedlings. Int J Mol Sci. 24: 6252.

Mirza JI, Bagni N. 1991. Effects of exogenous polyamines and difluoromethylornithine on seed germination and root growth of *Arabidopsis thaliana*. Plant Growth Regul. 10: 163-168.

Moumita, Mahmud JA, Biswas PK, Nahar K, Fujita M, Hasanuzzaman M. 2019. Exogenous application of gibberellic acid mitigates drought-induced damage in spring wheat. Acta Agrobot. 72(2): 1776.

Narayanan S . 2018. Effects of high temperature stress and traits associated with tolerance in wheat. Open Access J Sci. 2(3): 177–186.

Oh MH, Clouse SD. 1998. Brassinosteroid affects the rate of cell division in isolated leaf protoplasts of *Petunia hybrida*. Plant Cell Rep. 17: 921-924.

Öney S, Tabur S. 2013. Cytogenetical and molecular responses of exogenous potassium sulphate for tolerance to extreme temperatures in *Vicia faba* L. J Pure Appl Microbiol. 7: 663-670.

Öney S, Tabur S, Tuna M. 2015. Cytogenetic and molecular responses of ammonium sulphate application for tolerance to extreme temperatures in *Vicia faba* L. Cytol Genet. 49(5): 328-337.

Özmen S, Tabur S, Öney-Birol S, Özmen S. 2022. Molecular responses of exogenous polyamines under drought stress in the barley plants. Cytologia. 87(1): 7-15.

Özmen S, Tabur S, Öney-Birol S. 2023. Alleviation role of exogenous cadaverine on cell cycle, endogenous polyamines amounts and biochemical enzyme changes in barley seedlings under drought stress. Sci Rep. 13: 17488.

Patil BC, Bhat GI. 1992. A comparative study of MH and EMS in the induction of chromosomal aberrations on lateral root meristem in *Clitoria ternetea* L. Cytologia. 57: 259-264.

Pereira A. 2016. Plant abiotic stress challenges from the changing environment. Front Plant Sci. 7: 1123.

Powell JN, Grant CJ, Robinson SM, Radford SG. 1973. A comparison with halothane of the hormonal and anaesthetic properties of ethylene in plants. Br J Anaesth. 45(7): 682-690.

Pradhan SK, Gupta RC. 2013. Cytogenetic response of 24-epibrassinolide in *Brassica oleracea* var. *botrytis* under temperature stress. Crucif Newsl. 32: 11-13.

Prakash L, Prathapasenan G. 1990. Interactive effect of NaCl salinity and gibberellic acid and gibberellin like substances and yield of rice (*Oryza sativa* L. var. GR.3). Proc Indian Acad Sci. 100: 173-181.

Qureshi H, Abbas MH, Jan T, Mumtaz K, Mukhtar H, Khan U. 2022. Plant responses to heat stress. J Adv Nutr Sci Technol. 2(2): 40-53.

Rasheed R, Wahid A, Farooq M, Hussain I, Basra SMA. 2011. Role of proline and glycine betaine pretreatments in improving heat tolerance of sprouting sugarcane (*Saccharum* sp.) buds. Plant Growth Regul. 65: 35-45.

Sarwar M, Anjum S, Alam MW, Ali Q, Ayyub CM, Haider MS, Ashraf MI, Mahboob W. 2022. Triacontanol regulates morphological traits and enzymatic activi-

ties of salinity affected hot pepper plants. Sci Rep. 12: 3736.

Saxena S. 2022. Chromosomal aberrations in plant bioassays: A review. Plant Arch. 22(2): 327-330.

Shao J, Huang K, Batool M, Idrees F, Afzal R, Haroon M, Noushahi HA, Wu W, Hu Q, Lu X, Huang G, Aamer M, Hassan MU, El Sabagh A. 2022. Versatile roles of polyamines in improving abiotic stress tolerance of plants. Front Plant Sci. 13: 1003155.

Sharma SK. 2021. Brassinosteroids application responses in fruit crops – A review. Int J Agric Environ Biotechnol. 14(2): 123-140.

Sharma S, Singh V, Tanwar H, Mor VS, Kumar M, Punia RC, Dalal MS, Khan M, Sangwan S, Bhuker A, Dagar CS, Yashveer S, Singh J. 2022. Impact of high temperature on germination, seedling growth and enzymatic activity of wheat. Agriculture MDPI. 12: 1500.

Sheikhi S, Ebrahimi A, Heidari P, Amerian MR, Rashidi-Monfared S, Alipour H. 2023. Exogenous 24-epibrassinolide ameliorates tolerance to high-temperature by adjusting the biosynthesis of pigments, enzymatic, non-enzymatic antioxidants, and diosgenin content in fenugreek. Sci Rep. 13: 6661.

Shrestha S, Mahat J, Shrestha J, Madhav KC, Paude K. 2022. Influence of high temperature stress on rice growth and development. A review. Heliyon. 8: e12651.

Silveira GL, Lima MGF, dos Reis GB, Palmieri MJ, Andrade-Vieria LF. 2017. Toxic effects of environmental pollutants: Comparative investigation using *Allium cepa* L. and *Lactuca sativa* L. Chemosphere. 178: 359-367.

Sultan K, Perveen S, Parveen A, Atif M, Zafar S. 2023. Benzyl amino purine (BAP), moringa leaf extract and ascorbic acid induced drought stress tolerance in pea (*Pisum sativum* L.). Gesunde Pflanz. 75 (6): 2423-2436.

Tabur S, Demir K. 2008a. Effects of triacontanol pretreatment on mitotic index and chromosome abnormalities under salt stress. J Biol Sci Res. 1(1): 11-15.

Tabur S, Demir K. 2008b. Effects of combinations of some plant growth regulators on mitotic index and chromosomal aberrations of barley seeds germinated under saline (NaCl) conditions. Süleyman Demirel Univ Art Sci J Sci. 3(2): 162-173.

Tabur S, Demir K. 2009. Cytogenetic response of 24-epibrassinolide on the root meristem cells of barley seeds under salinity. Plant Growth Regul. 58: 119-123.

Tabur S, Demir K. 2010a. Role of some growth regulators on cytogenetic activity of barley under salt stress. Plant Growth Regul. 60: 99-104.

Tabur S, Demir K. 2010b. Protective roles of exogenous polyamines on chromosomal aberrations in *Hordeum vulgare* exposed to salinity. Biologia. 65: 947-953.

Tabur S, Yurtlu MD, Özmen S. 2019. Role of humic acid against salt induced cytotoxicity in *Hordeum vulgare* L. Caryologia. 72(3): 3-10.

Taheri M, Haghighi M. 2018. Benzyl adenine is more effective than potassium silicate on decreasing the detrimental effects of heat stress in pepper (*Capsicum annum* cv. PS301). Iran Agric Res. 37(1): 89-98.

Truta E, Zamfirache MM, Rosu C, Olteanu Z, Mihai C, Gherghel D. 2011. Cytogenetic effects induced by 2,4-D and kinetin in radish and common bean root meristems. Rom Agric Res. 28: 207-215.

Türkoğlu S. 2007. Genotoxicity of five food preservatives tested on root tips of *Allium cepa* L. Mut Res. 626: 4-14.

Tütünoğlu B, Aksoy Ö, Özbek R, Uçkan F. 2019. The effects of gibberellic acid on *Allium cepa* root tip meristematic cells. Biol Plant. 63: 365-370.

Ünal M, Palavan-Ünsal N, Tüfekçi MA. 2002. Role of putrescine and its biosynthetic inhibitor on seed germination root elongation and mitosis in *Hordeum vulgare* L. Bull Pure Appl Sci Sec B-Bot. 21: 33-38.

Verma T, Bhardwaj S, Singh J, Kapoor D, Prasad R. 2022. Triacontanol as a versatile plant growth regulator in overcoming negative effects of salt stress. J Agric Food Res. 10: 100351.

Wahid A, Farooq M, Rasheed R, Gelani S, Rasul E. 2010. Sugarcane under thermal stress: some biotechnological considerations. In: Kumar A (eds) Plant genetic transformation and molecular markers. Pointer Publishers, Jaipur, pp. 109-123.

Wahid A, Gelani S, Ashraf M, Foolad MR. 2007. Heat tolerance in plants: an overview. Environ Exp Bot. 61: 199-223.

Wang Y, Zhou Y, Wang R, Xu F, Tong S, Song C, Shao Y, Yi M, He J. 2022. Ethylene response factor LlERF110 mediates heat stress response via regulation of LlHsfA3A expression and interaction with LlHsfA2 in Lilies (*Lilium longiflorum*). Int J Mol Sci. 23: 16135.

Waraich EA, Ahmad R, Halim A, Aziz T. 2012. Alleviation of temperature stress by nutrient management in crop plants: A review. J Soil Sci Plant Nutr. 12: 221-244.

Wu YS, Yang CY. 2019. Ethylene-mediated signaling confers thermotolerance and regulates transcript levels of heat shock factors in rice seedlings under heat stress. Bot Stud. 60: 23.

Wu J, Lu Y, Zhou B, Hu Z. 2022. Effects of cytokinin and abscisic acid on heat resistance of *Vetiveria zizanioides.* Not Bot Horti Agrobot Cluj-Nap. 50(3): 12755.

Xu Y, Chu C, Yao S. 2021. The impact of high-temperature stress on rice: Challenges and solutions. Crop J. 9: 963-976.

Younis AA, Ismail HA. 2019. Triacontanol alleviated nickel toxicity in maize seedling by controlling its uptake and enhancing antioxidant system. J Adv Plant Biol. 1(3): 1-14.

Zaki MES, Shalaby MAF, Khalil IS, Abou-Sedera FA, Abd Allah MSA. 2014. Protective role of benzyladenine and putrescine on snap beans (*Phaseolus vulgaris* L.) productivity grown under high temperature. Middle East J Appl Sci. 4(4): 905-910.

Zandalinas SI, Balfagón D, Gómez-Cadenas A, Mittler R. 2022. Plant responses to climate change: metabolic changes under combined abiotic stresses. J Exp Bot. 73(11): 3339–3354.

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Physiological, genetical changes and *cdc2* gene expression for osmotic stressed *Vicia faba* reveal the alleviation effect of gamma radiation and putrescine

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Abstract. Climate change caused increasing in soil salinity worldwide. Therefore, it is critical to enhance the capability of plant to tolerate salinity stress. For this goal, putrescine and irradiation by gamma radiation were used to improve the salt tolerance of *Vicia faba* (the most important human crop). The results indicated depression in mitotic division and all growth parameters associated with the induction of micronucleus (MN) when salinity increased to 100mM, while there was increase in mitotic aberrations. NaCl decreased total soluble sugar, while total N%, total free amino acid, proline and protein contents showed slight increase with increasing salinity stress. Putrescine and gamma radiation mitigated the effect of salinity on cell division and growth parameters. Salt stress decreased the expression of gene encoding cyclin-dependent kinase 2 (*cdc2*). Both putrescine and gamma radiation increased *cdc2* expression. The genetic diversity has been detected among control and treated *V. faba* using ISSR and SCoT markers. Ten primers had successfully generated 129 reproducible polymorphic amplicons that were suitable for studying the genetic diversity between studied genotypes. ISSR markers provided more discriminating data and were more informative than SCoT markers. Besides, cluster analysis using UPGMA and PAC successfully explained the genetic diversity within studied genotypes. These findings emphasize the efficiency of putrescine and gamma radiation for alleviating the negative impact of salt stress. Moreover, prove the importance of assessing mitotic activity, chromosomes behavior, physiological parameters, the expression level of *cdc2* and molecular diversity in *V. faba* under stress to improve the salt tolerance of it.

Keywords: *Vicia faba*, salt stress, putrescine, gamma radiation, growth parameters, cell division, mitotic aberrations, micronucleus, *cdc2* expression, ISSR& SCoT.

INTRODUCTION

The significant threats in the world today are caused by climate change. In the twenty-first century, salinity is one of the key issues brought on by cli-

mate change and one of the greatest risks to plants and crop yields globally, its negative impacts are increased under the severe changes in climate, particularly in arid and semiarid regions. According to recent statistics, salt stress has abused more than 45 million hectares of irrigated land worldwide, and this number is always rising (Isayenkov and Maathuis 2019). Additionally, salt stress generates a significant amount of reactive oxygen species, which damage some molecules such as lipids, proteins, and DNA, cause breakdown of cell membrane systems and terminate some enzymatic processes (Demiral and Türkan 2005). Plants use several mechanisms to manage salt damage and regulate cellular homeostasis and growth to overcome this problem. According to Isayenkov and Maathuis (2019), salt tolerance frequently involves the activation of cell signaling pathways that result in the production of antioxidant enzymes, and osmo-protective metabolites such amino acids and carbohydrates.

Vicia faba L. (2n = 12) or faba bean is one of the most important leguminous crops produced worldwide. Faba bean is the fourth most significant leguminous plant in the world (FAOSTAT 2018). Approximately 2.56 million hectares of land are harvested each year to yield 4.56 million tons of dry *V. faba* grains (FAOSTAT 2022). Eighty percent of the dried faba bean grains are produced in Asia and Africa (FAOSTAT 2022). In the Middle East, North Africa, the Mediterranean region, the Nile Valley, and Ethiopia, *V. faba* is regarded as a crucial food crop for human nutrition and cattle feed because mature *Vicia faba* seeds are a excellent source of protein, carbohydrates, cellulose, vitamin C, and minerals (Qahtan et al. 2021).

Polyamines (PAs) play a crucial role during environmental stress. They are low molecular weight growth regulators and present as aliphatic amines. According to FAO Statistics (2021) polyamines are multifunctional polycationic plant growth regulators that have an impact on several physiological, metabolic, and developmental processes. Wisniewski et al. (2014) reported that PAs could control DNA replication, cell division, seed germination, and development. Putrescine, spermidine, and spermine are typical polyamines found in plants (Gupta et al. 2013). One of the main polyamines, putrescine, is crucial for plant growth and differentiation as well as stress responses (Sequera-Mutiozabal et al. 2017). The positive charges of PAs can help stabilize cell membranes under environmental stress by attaching to the negatively charged phospholipids and proteins that make up the membranes (Kuznetsov and Shevyakova 2007), Additionally, PAs enhance antioxidant systems, regulate some gene expression (Matkovics et al. 1993) and cause scavenging of free radicals (Velikova et al. 2000).

Useful mutations are the modifications of the genotypic structure to improve the species' variability and help them to respond better to different range of stresses (Spencer et al. 2018). Physical mutagenic agents including ultraviolet light, protons, neutrons, alpha and beta particles, and ionizing radiation (X-rays and gamma rays) can cause useful mutations. Gamma radiation can directly cause physical, biological, and chemical changes in cells (Ludovici et al. 2020). It can indirectly affect free radical production and directly trigger specific alterations in the genome (Caplin and Willey 2018).

Molecular markers are considered an effective method for analyzing and identifying genetic variability within and/or between genotypes. The degree of polymorphism affects how discriminatory power, which in turn determine how markers are categorized. To ascertain the genetic diversity among species, cultivars, and treatments, polymorphism is used. In recent decades, research on the genetic diversity of genotypes has been used in numbers of disciplines, including genetics, ecology, botany, biology, and others (Chesnokov and Artemyeva 2015). Inter-Simple Sequence Repeat (ISSR) markers are Polymerase Chain Reaction PCR (PCR) approaches for measuring the genetic diversity in plants (Ziêtkiewicz et al. 1994). These markers amplify the inter-SSR sequences of varied sizes. Start codon targeted (SCoT) was generated to start a trend away from random DNA markers and toward gene-targeted markers based on the short on served region flanking the ATG of plant genes. Since the SCoT marker is frequently reliable, it is understood that factors other than annealing temperature and primer length can affect repeatability (Collard and Mackill 2009). Since no specific knowledge of the genome sequence was required for the SCoT markers design, it was possible to apply it to plants without genome references (Xiong et al. 2011). In *V. faba* and many other plant species, both markers are frequently used to assess genetic diversity (Albrifcany et al. 2022).

The most vulnerable stages of growth, germination, and seedling development are partially affected by cell cycle suppression. Salinity reduces the cell division frequency. It also causes defects in the chromosomes structure and caused induction of micronuclei (Souguir et al. 2018). Micronucleus Test (MN Test) is used to evaluate the genotoxic potential of substances.

Biotic and abiotic stress cause cell cycle inhibition which in turn causes harmful effect on plant growth. Although the molecular interactions that link the cell cycle machinery to perception of stress are not fully understood, recent studies indicated the involvement of cyclin dependent kinases (cdcs) in the plant response

machinery (Kitsios and Doonan 2011). Cell divisions in eukaryotic cells are controlled by a family of protein kinases, the cyclin-dependent kinases (cdcs). The activity of cdcs is regulated by cyclins, or to the cdc inhibitors. The activity and localization of different cdc complexes regulate many of the actions during the cell cycle (Nigg 1995). In plants and animals many cdcs are present and regulate the G_1/S and the G_2/M transitions (Magyar et al. 1997). Different plant species contain one or two cdc gene, which contains a fully conserved PSTAIRE sequence motif (Hirt and Heberle-Bors 1994). Mutant cdc gene in *Arabidopsis (cdc2a-At)* arrest cell cycle at G_1/S and/or G_2/M points, demonstrating its ability to control both checkpoints (Hemerly et al. 1995).

From the previous, plants must develop mechanisms to adapt to the variable environment conditions. Therefore, this study was conducted to declare the role of gamma radiation and putrescine in the alleviation of the harmful effect of salinity on *V. faba* plants by tracking the physiological, genetical, and molecular changes and affirm the involvement of *cdc2* in the signaling control of stress tolerance.

MATERIALS AND METHODS

Seeds of bean (*Vicia faba*) (cv. Giza 2) were obtained from the Agriculture Research Center, Ministry of Agriculture, Giza, Egypt.

Radiation treatments

Dry seeds irradiation was performed at National Center for Radiation Research and Technology (NCRRT). Cairo, Egypt. By using (Co60) as source of γ-rays at dose levels 10 Gy with dose rate of 0.623 rad/sec.

Putrescine treatments

Putrescine solutions were prepared at levels of the solutions (5 mMol) were sprayed on leaves of the seedlings after the appearance of first leave and repeated every seven days until harvest after 50 days.

Experimental design

A pot experiment was conducted under field conditions at a wire house at NCRRT, Cairo, Egypt. The seeds were irradiated with 10 Gy radiation dose and then three seeds were grown in each plastic pot, (40 × 35 cm, height × diameter), containing equal quantities of sandy loam soil, commercial peat and clay. The seeds were left to grow inside the greenhouse under natural lighting. A full concentration of Hoagland's nutrient solution was used to irrigate pots (Hoagland and Arnon, 1950).

Randomized complete block design with three replications was used for the experimental design. Salt solution (250 ml) was applied at 15 days after seedling emergence twice per weak. Foliar spray of putrescine was applied after 2day from salinity treatments. It repeated twice a week.

Either:

1) Full strength Hoagland's solution (control).
2) Full strength Hoagland's solution +50 mMNaCl.
3) Full strength Hoagland's solution + 100 mM NaCl.
4) Full strength Hoagland's solution + 5 mM putrescine (foliar spray).
5) Full strength Hoagland's solution + 5 mM putrescine (foliar spray) + 50 mM NaCl.
6) Full strength Hoagland's solution + 5 m M putrescine (foliar spray) + 100 mM NaCl.
7) Full strength Hoagland's solution + 10 Gy.
8) Full strength Hoagland's solution + 10 Gy + 50 mM NaCl.
9) Full strength Hoagland's solution + 10 Gy +100 mM NaCl.

After 50 days seven growth parameters were measured.

Physiological analysis

Extraction of plant extract. Fresh grinded leaves (0.1g) were accurately weighted then extracted by in 80 % aqueous ethanol for 6 h (AOAC 1984). The extract was filtered and completed to 50 ml in a measuring flask with ethanol 80% for further experiments.

Determination of total soluble sugar. Total soluble sugar of the fresh leaves was quantified using a modified phenol-sulfuric acid assay (Zhang 1993).

Determination of total free amino acid. Total free amino acid of fresh leaves was measured according to Rosen (1957).

Determination of proline content. Proline content was evaluated using the method of Bates et al. (1973).

Determination of Nitrogen (N) %. The leaf N concentration (LNC) was assessed according to the method Wild et al. (1985).

Determination of total soluble protein content. Total soluble proteins were estimated according to Lowery et al. (1951).

Genetical analysis

Quantitative analysis of mitotic division and chromosomal aberrations. To get rid of the soil remnants, the roots of each sample group were taken and rinsed under running water. Root tips (3 to 4 cm in length) were stored in 70% ethyl alcohol at 4°C until usage after being treated in Carnoy's solution for 24 hours. After being hydrolyzed in 1N HCl, the roots were stained for one hour with basic Fuchsin stain. The root tips that were darkly discolored were crushed in a drop of 45% acetic acid. For each treatment group and control group, 1000 cells on 10 slides were used to score and compute the percentage of mitotic division (MI) and frequency of mitotic abnormalities (Xavier et al., 2023).

Quantitative analysis of micronucleus. Cell with micronuclei (MN) reveals the genotoxicity effect of treatment. Cells with intact cell wall that contains MN alongside the nucleus were scored. The frequency of MN was scored in 5000 cells and expressed as the percentage of the number of cells with micronuclei per 1000 cells per slide (Xavier et al. 2023). MN was scored to prove genotoxic effect of salinity stress and the mitigating effect of both putrescine and gamma radiation.

*Cyclin dependent kinase 2 (*cdc2*) gene expression quantification using qReal-Time PCR.* Pure RNA was extracted from *V. faba* root for all experiment groups using total RNA Purification Kit (Qiagen, Rneasy Kit) following the manufacturer protocol. RNA input for all samples was adjusted to 1000 µg and cDNA was synthesized using Reverse Transcription kit (Thermo Fisher Scientific, RevertAid RT Reverse Transcription Kit). Specific primers for *cdc2* gene F 5'ACTCTCATAGGGTTCTCC3' (Tm 54°C); R 5' CTCGGTACCAGAGAGTAA3' (Tm 54°C) were used. The amplification protocol was as follow, 1 cycle at 95°C for 10 min followed by 40 twostep cycles 95 °C for 15 sec, and 60 °C for 60 sec. Results were normalized by housekeeping *actin* gene using the following primer F 5' CTTCCCAAGATAGTAGGAG3' (Tm 55°C) and R 5' CTTAGACTGTGCCTCATC3' (Tm 54°C). The expression level of genes was calculated in relation to $2^{-\Delta\Delta ct}$ according to Livak and Schmittgen (2001).

Molecular fingerprint using ISSR-PCR and SCoT-PCR. DNA was isolated from *V. faba* fresh leaves according to cetyltrimethylammonium bromide (CTAB) method. The quality of DNA was checked at 260/280 nm and on 1% agarose gel. DNA was used for ISSR and SCoT techniques according to Ziêtkiewicz et al. (1994) and Collard and Mackill (2009) respectively. Ten primers (Table1) five for each technique were screened against each DNA sample to analyze molecular diversity among all treated and control groups. Polymerase chain reaction (PCR) was accomplished in an automated thermal cycle (model Techno 512, Stafford, UK system) using Dream Taq green master Mix (Thermo-Scientific). The PCR reaction volume was 25 µl including 2.5 µl of dNTPs (2.5 mM), 1.5 µl of $MgCl_2$ (25 mM), 2.5 µl of 10× buffer, 2.0 µl of primer (2.5 µM), 2.0 µl of template DNA (50 ng µl/1), 0.3 µl of Taq polymerase (5 U µl/µ1) and 14.7 µl of sterile ddH_2O. PCR reaction for both fingerprints was 1 cycle at 95°C for 5 min followed by 40 cycles of 1 min at 95°C, 1 min at annealing temperature 56°C and 2 min at 72°C, followed by 1 cycle at 72°C for 10 min. PCR products were resolved on a 1.5% agarose gel with 100 – 3000bp DNA Ladder, GeneDirex, Inc (100bp DNA Ladder H3 RTU Ready-to-Use).

Table 1. ISSR and SCoT primers nucleotide sequences.

Analysis	Primer	Nucleotide sequence of the used primer 5' to 3'
ISSR	UBC810	GTGTGTGTGTGTGTGTCA
	UBC818	CAC ACA CAC ACA CAC AG
	UBC849	GAGAGAGAGAGAGAGAT
	UBC-823	TCTCTCTCTCTCTCTCC
	UBC-817	CACACACACACACACAA
SCoT	SCoT -31	CCATGGCTACCACCGCCT
	SCoT -34	ACCATGGCTACCACCGCA
	SCoT -13	ACGACATGGCGACCATCG
	SCoT -14	ACGACATGGCGACCACGC
	SCoT -52	ACAATGGCTACCACTGCA

Data and cluster analyses

Binary data matrix for the bands (0 for absent 1 for present bands) were analyzed. Polymorphism percentage, total number of amplicons (TNA), total polymorphic amplicons (TPA), monomorphic amplicons (MA) and unique amplicon (UA) were scored. Four parameters, polymorphic information content (PIC), effective multiplex ratio (EMR), marker index, and resolving power (RP) were examined the efficiency of ISSR and SCoT markers as well as the genetic difference among genotypes under study. PIC, Rp, EMR and MI were calculated according Venkatesan et al (2021).

The genetic similarity coefficient was used to construct the phylogenetic tree. Genetic similarity coefficient between two genotypes and principal component analysis (PCA) was achevied using the Paleontological Statistics Software Package for Education and Data Analysis (PAST) version 4.03 (Hammer et al. 2001).

Statistical analysis of data

Statistical analysis was carried out according to Snedecor and Cochran (1980). The individual comparisons between the obtained data were carried using M-STAT computer software program at $p \leq 0.05$.

RESULTS

Physiological analysis

Figure 1A declared the effect of salinity, putrescine, and gamma radiation on the stem length of *V. faba* plants. Increasing NaCl concentration from 50 mM to 100 mM decreased stem length from 33.71 to 31.11 cm compared to the control (36.50 cm). Foliar spraying with putrescine increased stem length to (37.72 cm) compared to the control plants. It is obvious that gamma radiation gave the longest stem length (48.68 cm) above all treatments used especially with 10Gy + 50 mM NaCl treated plants. The same trend was observed with the root length i.e. salinity reduced root length to 2.4 cm with 100m M NaCl treated plants. Putrescine and radiation alleviated the harmful effect of salinity as they gave 4 cm with 5m M treated plants and 6.5 cm with 10 Gy + 50 m M NaCl treated plants. In Fig. 1C number of leaves decreased to 12 leaves with increasing salinity concentration to 100 mM. While putrescine and radiation had a non-significant effect on the number of leaves. On the other hand, radiation increased shoot fresh and dry weight above the control or putrescine treatments (Fig. 1 D, E). The maximum shoot fresh and dry weight was 36.8 g and 7.1 g in 10 Gy + 50 Mm NaCl treated plants respectively. The same effect of radiation appeared in root fresh weight and dry weight (Fig. 1 F, G). Figure 2A showed the effect of salinity, putrescine, and radiation on total soluble sugars (TSS) of *V. faba* plants. It is cleared that salinity reduced TSS% from 1.89% to 1.52% as salinity increased from zero to 100 Mm NaCl. Foliar spraying of putrescine has a positive effect in TSS % i.e increased to 2.64%with 5 mM put + 50 mM NaCl treated plants. The highest TSS% was observed with radiation in all treatments used especially with 10 Gy+50 mM NaCl treated plants (2.508%). Fig. 2B cleared the influence of salinity on total free amino acid. The maximum increment (0.1221g/ 100 g) reached by 100 mM NaCl treated plants. Radiation also increased the total amino acid with 10 Gy + 100 mM NaCl treated plants (0.2089 g/ 100 g).

Salinity showed positive effect on proline contents of leaves, the content of proline increased as salinity increased, it reached 7.004 mg/100g in plants treated with 100 mM NaCL compared to zero NaCl 2.8600 mg/100g. This increment is still observed with putrescine treatments. There was a decrease in proline content (7.5840) by 10Gy +100mM NaCl than with 10 Gy treated plants (9.278). Concerning N% contents in leaves of *V. faba* plant, N% increased in 50 mM NaCl treated plants and 10 Gy + 50 m M NaCl treated plants to 0.407% and 0.438% respectively. Fig. 2E illustrates that protein content increased with increasing salinity concentration, the maximum increase observed with 50 mM NaCl treated plants. Putrescine and radiation increased protein contents of *V. faba* leaves. Treatment with both 50 mM NaCl+ 5mM putrescine and 50 mM + 10 Gy showed the maximum value of protein contents (1.56 and 2.74 respectively).

Genetical analysis

Quantitative analysis of mitotic division and chromosomal aberrations

The mitotic index (MI) is a very important indicator for the rate of mitotic division. Salinity stress by 50mM and 100mM NaCl caused significant decrease in MI of *V. faba* meristematic root cells reached 5.58±0.14 and 4.98±0.20 respectively as compared with control (Fig. 3A). Alleviation effect of both putrescine and gamma radiation (at low dose 10 Gy) was obvious specifically with concentration of 50mM NaCl. Foliar application of salt stressed *V. faba* with 5mM putrescine caused highly significant increase in MI as compared with salinity stressed *V. faba,* but MI was still less than control. While gamma radiation mitigated the harmful effect of NaCl and enhanced the mitotic division and caused highly significant increase in MI as compared with control and salt stressed plants. The maximum MI was 7.84±0.21, which has attained after treatment with 10 Gy + 50mMNaCl.

The changes in mitotic index frequencies were associated with significantly increment in mitotic aberrations percentage under salinity stress. The frequency of mitotic abnormality after treatment with 50mM NaCl was 20.64±0.75, while the ratio of mitotic abnormality after treatment with 100mM NaCl was 31.21±0.26. As well as all treatments with gamma radiation and putrescine significantly increased the ratio of mitotic abnormality but less than those of salt stress treatment (Fig. 3B). Only one exception, treatment with 10 Gy with 100mM NaCl caused higher frequency of mitotic aberration reached 32.44±0.39 (the maximum ratio), while the minimum ratio was produced after the foliar application by putrescine only.

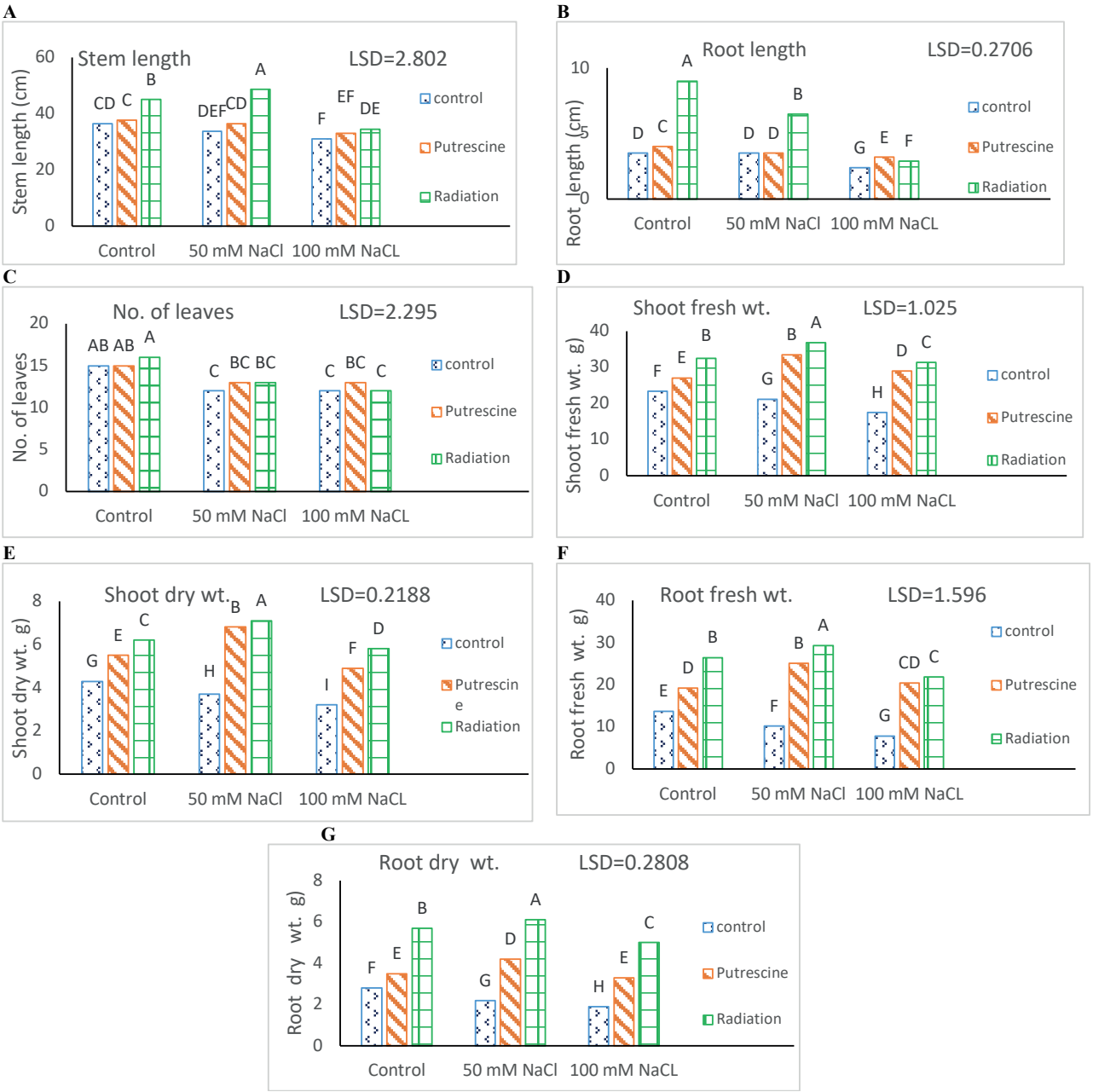


Figure 1. Effect of salinity, putrecine and gamma radiation on growth of bean plant (Non identical letters indicate significant difference).

Additionally, the reduction of MI after salinity stress was accompanied with significant increase in prophase index (Fig. 3C). The maximum prophase accumulation was 48.16±0.14 achieved by treatment with 100mMNaCl. Concerning treatment with putrescine, phase indices showed non-significant accumulation of prophase and displayed normal contribution of phases as compared with control. While treatment with gamma radiation reflected general accumulation in metaphase. The maximum metaphase accumulation was 42.97±0.23 achieved by treatment with 10 Gy.

Several types of chromosomal aberrations were observed after all treatments as compared with control. There were three main classes of chromosomal abnormalities (Fig. 4i-xii). The first class was chromosome stickiness, the second class was clastogenic aberrations including breakage, bridge, and ring chromosome, and finally chromosome disturbance including disturbance,

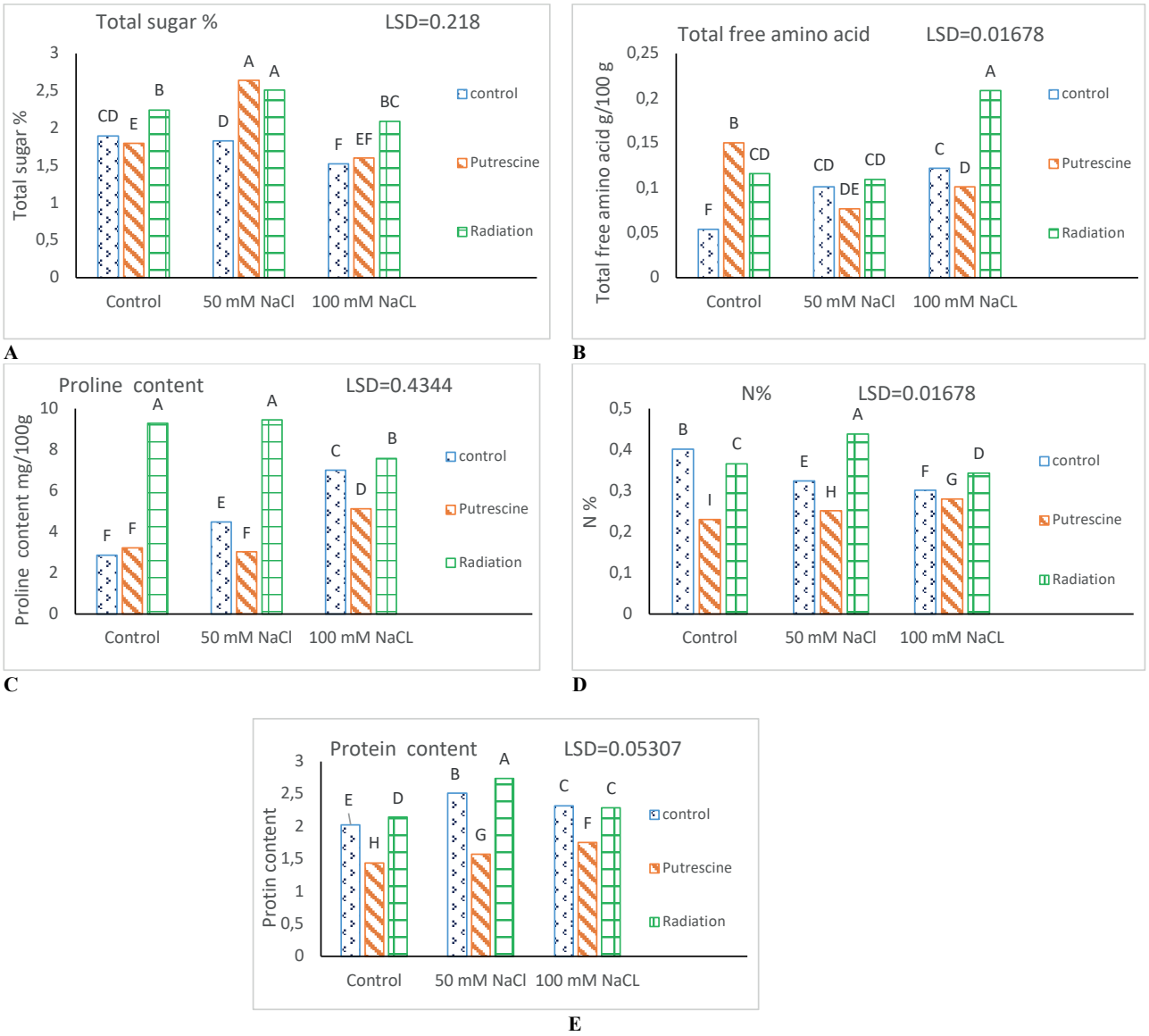


Figure 2. Effect of salinity, putrescine and gamma radiation on physiological contents of bean plants (Non identical letters indicate significant difference).

multipolarity, diagonal. Chromosome stickiness was more pronounced by salt stress. Although the alleviation effect of gamma radiation on MI, clastogenic aberration was more obvious after gamma radiation treatment.

Quantitative analysis of micronucleus

Meristematic cells of *V. faba* under salt stress and gamma radiation showed significant production of MN. The formation of MN was significant at level $p \leq 0.001$. Micronucleus was observed in interphase cells and in several phases (Fig. 4 xiii-xvi). The percentage of MN formation was concentration dependent. Treatment with 10 Gy+100mM NaCl exhibited the highest frequency of MN (18.46±0.11). Foliar application of putrescine alleviated the toxic effect of NaCl and produced the least MN percentage 3.47±0.26 after treatment with 5mM putrescine (Fig. 5).

Cyclin dependent kinase 2 (*cdc2*) gene expression quantification using qReal-Time PCR

Significant changes were recognized in the expression level of *cdc2* gene in *V. faba* root. Marked sig-

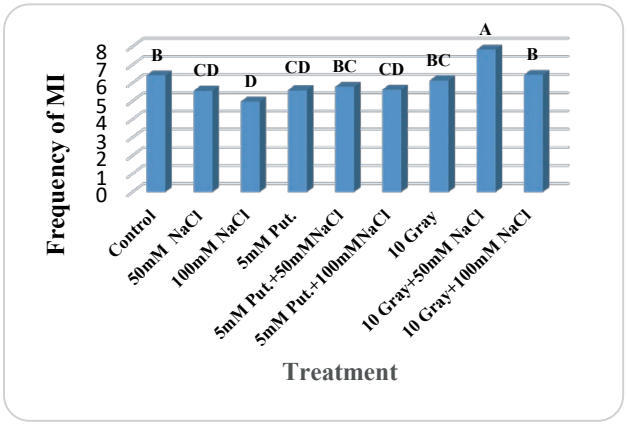


Figure 3A. Change in mitotic index of osmotic stressed *V. faba* treated by gamma radiation and putrescine. Non identical letters indicate significant difference at $p \leq 0.001$ LSD= 0.6671.

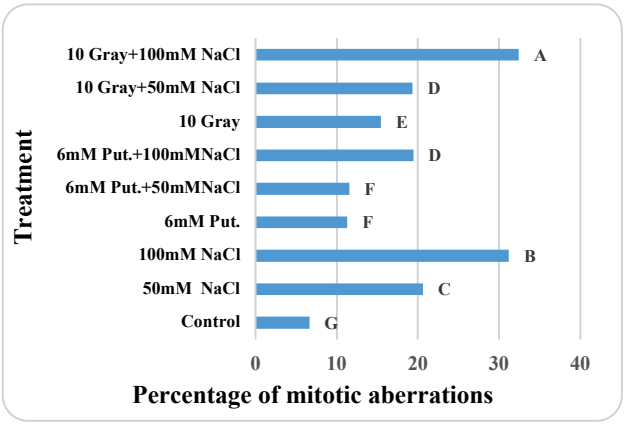
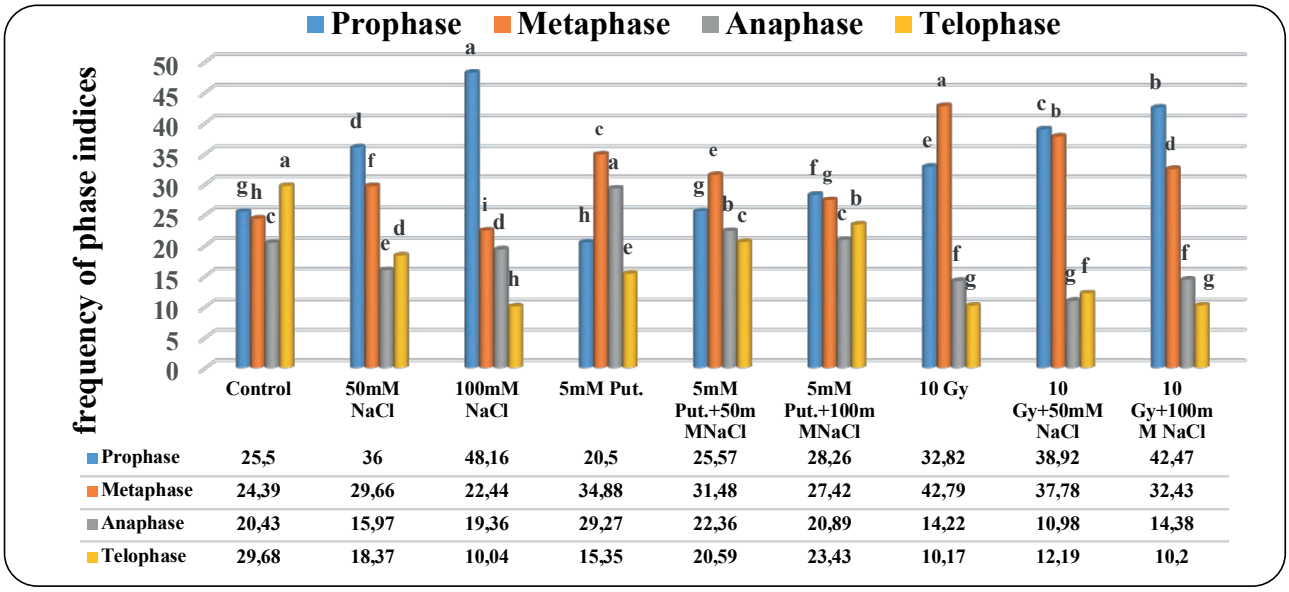


Figure 3B. Frequency of mitotic abnormalities of osmotic stressed V. faba treated by gamma radiation and putrescine. Non identical letters indicate significant difference at $p \leq 0.001$ LSD=0.7180.



	Control	50mM NaCl	100mM NaCl	5mM Put.	5mM Put.+50mMNaCl	5mM Put.+100mMNaCl	10 Gy	10 Gy+50mM NaCl	10 Gy+100mM NaCl
Prophase	25,5	36	48,16	20,5	25,57	28,26	32,82	38,92	42,47
Metaphase	24,39	29,66	22,44	34,88	31,48	27,42	42,79	37,78	32,43
Anaphase	20,43	15,97	19,36	29,27	22,36	20,89	14,22	10,98	14,38
Telophase	29,68	18,37	10,04	15,35	20,59	23,43	10,17	12,19	10,2

Figure 3C. Change in mitotic phases of osmotic stressed *V. faba* treated by putrescine and gamma radiation. Non identical letters for each phase indicate significant difference at $p \leq 0.001$.

nificant down regulation was recorded after treatment with 50 and100mM NaCl (Fig. 6). Conversely, marked upregulation in *cdc2* expression level was noticed after the combination between NaCl and putrescine or with10Gy radiation. Exposure of *V. faba* to 5mM putrescine+50mM NaCl and 10Gy+ 50mM NaCl increased the cdc2 expression level 2-folds and 4-fold respectively. In contrast, both 5mMputrescine and 10Gy with 100mM NaCl caused downregulation of *cdc2* gene expression level (0.965 and 1.04 respectively), which were more than those of salt stressed only.

Molecular fingerprint using ISSR-PCR and SCoT-PCR)

ISSR and SCoT markers were used to investigate the molecular variations in osmotic stressed *V. faba* treated with putrescine as well as gamma radiation. Ten ISSR and SCoT-primers (5 for each analysis) succeeded in amplifying 126 amplicons with a range between 2840 to 135 bp (Fig.7). Eighty amplicons of 126 were reproducible polymorphic amplicons, 25 amplicons of 80 were unique amplicons, while 46 were reproducible monomorphic amplicons. The maximum total number of

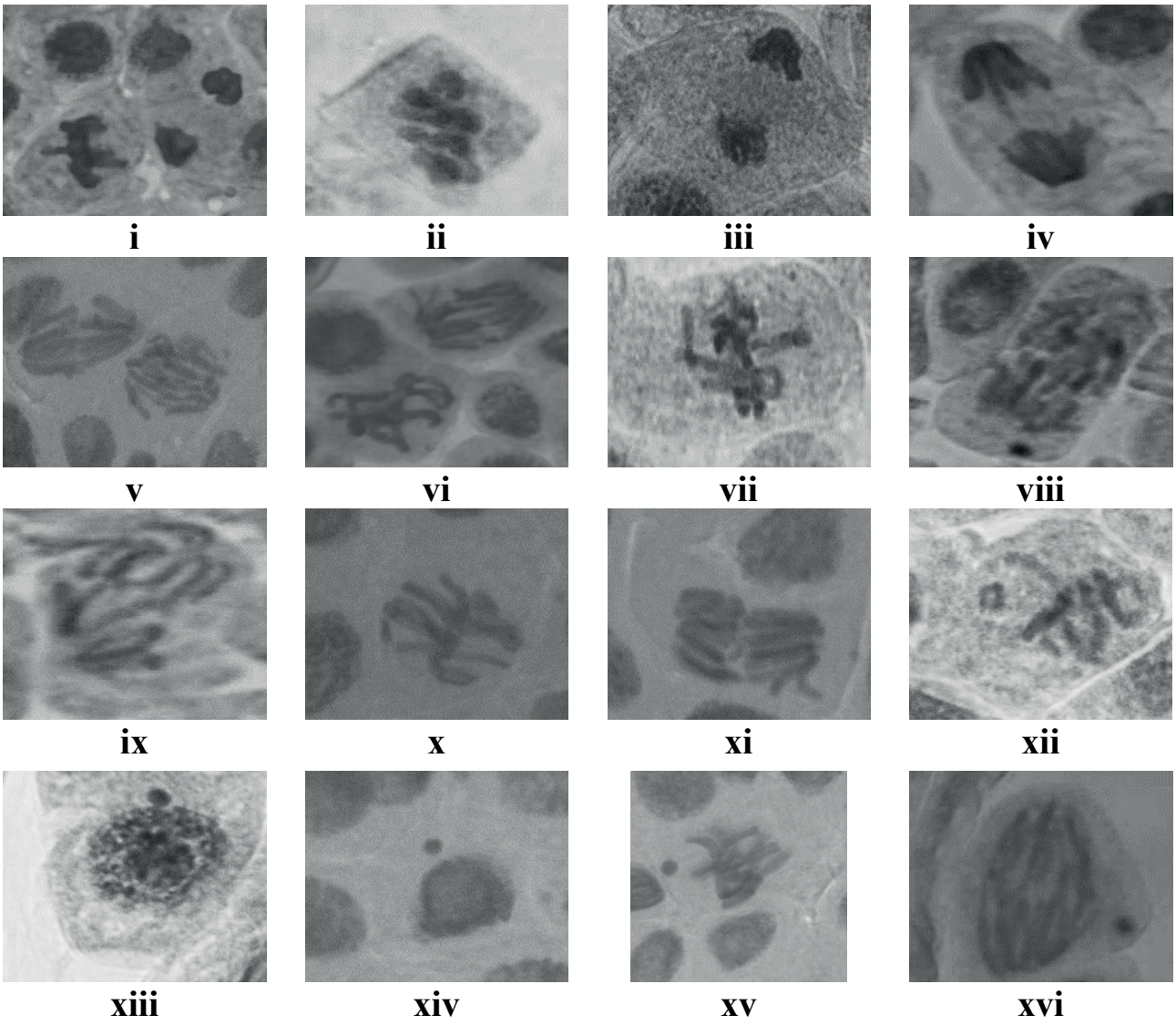


Figure 4. Types of chromosomal aberrations formed in *V. faba* by different treatments. i: Sticky metaphase and anaphase, ii: Sticky metaphase, iii: Sticky anaphase, iv: Sticky anaphase with bridge, v: Disturbance anaphase with multibridge, vi: Disturbance anaphase with laggard, vii: Disturbance metaphase, viii: C-anaphase, ix: Multipolarity, x: Metaphase with laggard, xi: Anaphase with laggard, xii: Metaphase with ring chromosome, xiii-xiv: Interphase with micronucleus, xv: Metaphase with micronucleus, xvi: Anaphase with micronucleus.

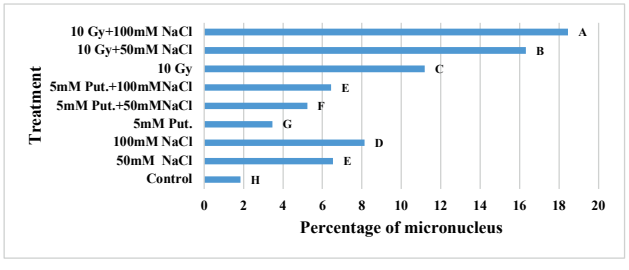


Figure 5. Frequency of micronucleus in osmotic stressed *V. faba* treated by putrescine and gamma radiation. Non identical letters indicate significant difference at $p \leq 0.001$ LSD=0.1914.

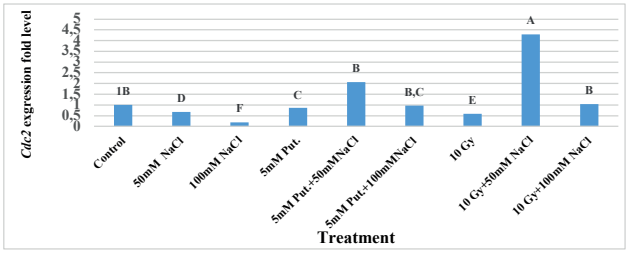


Figure 6. Change in *cdc2* gene expression level of osmotic stressed *V. faba* treated by putrescine and gamma radiation. Non identical letters indicate significant difference at $p \leq 0.001$ LSD=0.9360.

Table 2. ISSR-PCR and SCoT-PCR amplicons from osmotic stressed *V. faba* treated by putrescine and gamma radiation.

Analysis	Primer	Range of amplicon molecular size (bp)	TNA	MN	TPA	UA	Polymorphism %	PIC	RP	EMR	Marker index
	UBC810	1480-170	9	3	6	2	66.66%	0.61	2.64	4	2.5
	UBC818	2050-188	10	4	6	2	60.00%	0.10	4.22	3.6	0.36
	UBC849	1600-155	10	5	5	3	50.00%	0.80	1.82	2.5	2
ISSR	UBC-823	1960-160	14	6	8	4	57.14%	0.10	4.70	4.9	0.45
	UBC-817	2250-275	13	5	8	4	61.53%	0.64	2.96	4.9	3.15
		Total	56	23	33	15		2.25	16.34	19.9	8.46
		Mean	11.2	4.6	6.6	3	59.07%	0.45	2.27	3.98	1.69
	SCoT -31	2840-135	17	8	9	0	52.94	0.16	6.16	4.76	0.76
	SCoT -34	2050-233	18	3	15	0	83.33	0.20	10.55	12.5	2.5
	SCoT -13	2090-320	13	6	7	4	53.84	0.65	2.72	3.77	2.45
SCoT	SCoT -14	1935-310	13	3	10	3	76.92	0.15	4.68	8.10	1.22
	SCoT -52	2100-240	9	3	6	3	66.66	0.09	5.14	7.69	0.69
		Total	70	23	47	10		1.25	29.25	36.82	7.62
		Mean	14	4.6	9.4	2	66.74	0.25	5.85	7.36	1.52

amplicons was 18 amplicons for primer SCoT-34 with 83.33 % percentage of polymorphism, while the minimum number of amplicons was 9 produced by both primers UBC810 and SCoT-52 with 66.6% polymorphism for the two primers. The minimum percentage of polymorphism was 50% achieved by UBC849 primer (Table 2).

ISSR and SCoT markers performance

The polymorphism information contents (PIC), or heterozygosity index displays the capability of each marker for revealing the frequency of polymorphism or genetic diversity between the different genotypes. It was calculated for each locus depending on the number of alleles and the allele frequency. Table 2 revealed that PIC values of ISSR markers was ranged from 0.10 to 0.80 with average of 0.45, which reflected intermediate level of polymorphisms for the used ISSR sites in the different treated genotypes of *V. faba*. Three primers (UBC810, UBC849 and UBC-817) were highly informative, PIC value > 0.5 and two primers (UBC818 and UBC-817) were low informative, PIC value < 0.25. While The PIC value of SCoT markers ranged from 0.09 to 0.65 with average of 0.25, which reflected low level of polymorphisms. Four primers were low informative, PIC value < 0.25 and only one primer SCoT -13 was highly informative, PIC value > 0.5 reached 0.65.

The resolving power (Rp) is the most effective parameter used to discriminate effectiveness of the primer to reveal genetic diversity level among individuals. Rp value of ISSR was ranged from 1.82 (UBC849) to 4.70 (UBC-823), Rp value of SCoT was ranged from 2.75(SCoT-13) to 10.55 (SCoT-34).

Table 2 shows that UBC-823 generated the highest number of amplicon (14) and highest RP value (4.70) with 57.14% polymorphism frequency, as well primers SCoT-34 targeted the highest number of amplicons (18) with a polymorphism percent of 83.33% and highest RP value (10.55). This demonstrates that these two primers (UBC-823 and SCoT-34) had high-edifying and discriminative abilities in determining genetic diversity.

EMR was calculated as the total number of polymorphic loci for each primer. Marker is more efficient when the EMR value was higher. SCoT-34 gave the highest EMR value (12.5) among all used ISSR and SCoT primers (Table 2).

Marker index is a statistical tool to describe the capability of each primer to discriminate polymorphic loci among the used genotypes. The maximum marker index value was 3.15 achieved by ISSR marker UBC-817 (Table 2).

Treatment specific ISSR and SCoT primer

Twenty positive marker amplicons were produced by the 10 loci of ISSR and SCoT. All ISSR primers were successful in generating 13 amplicons extending from 260 to 1600 pb, while 3 SCoT primers (SCoT-13, SCoT-14, and SCoT-52) were able to target 7 amplicons ranging from 515 to 2090 (Table 3). These primers could be considered as marker related specific treatments.

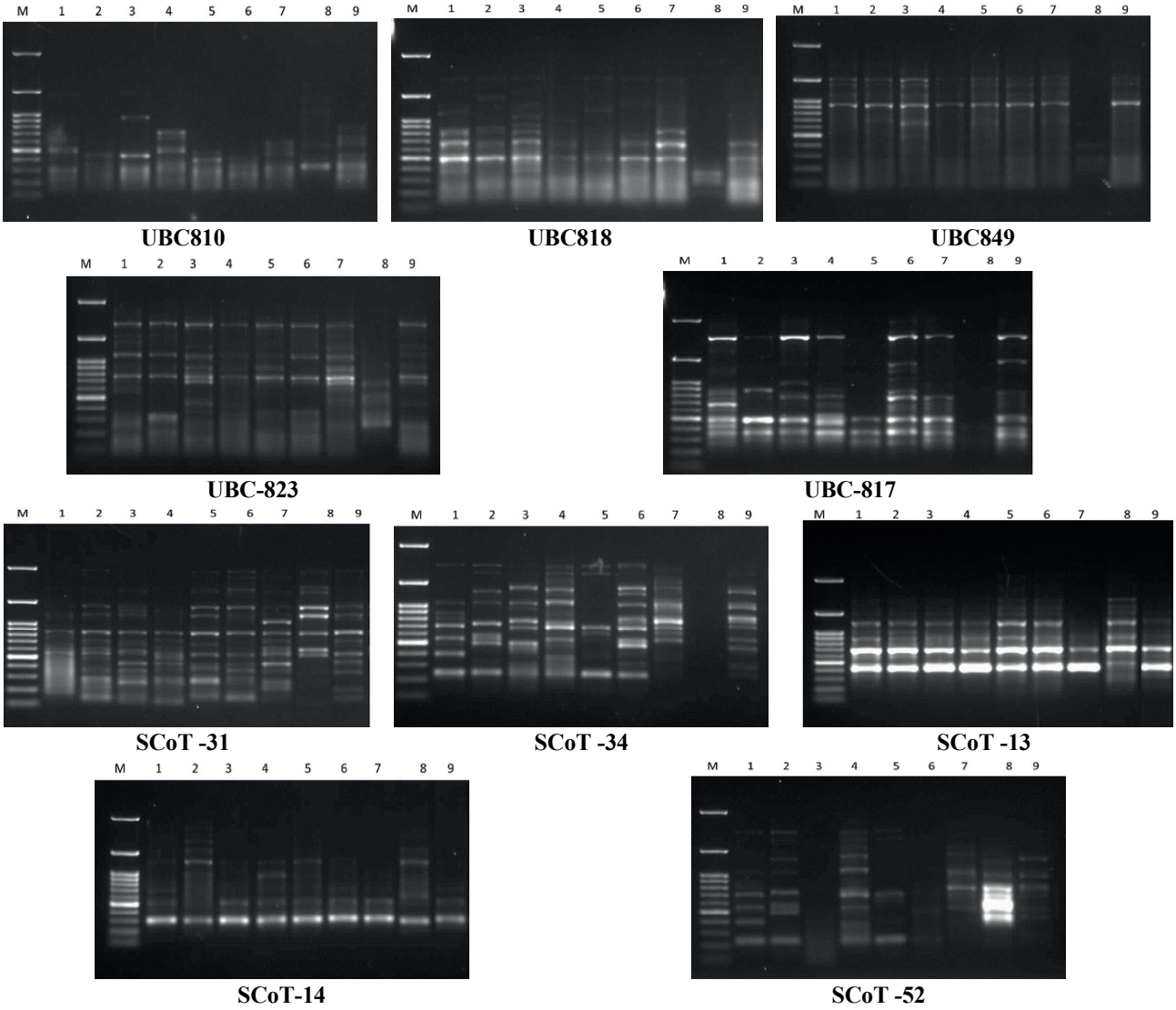


Figure 7. ISSR-PCR and SCoT-PCR profiles from osmotic stressed *V. faba* treated by putrescine and gamma radiation. M: Marker, 1:Control, 2:50mmNaCl, 3:100mM NaCl, 4:5mM Put., 5:5mM Put.+ 50mMNaCl, 6:5mM Put.+ 100mMNaCl, 7:10Gy, 8:10Gy+ 50mMNaCl, 9: 10Gy+ 100mMNaCl.

Cluster analysis

The dendrogram generated using UPGMA depends on ISSR and SCoT data revealed that all treatments and control form one cluster (cluster I), only radiated plants with 100NaCl formed a distinct cluster (cluster II) (Fig 8A). The first cluster separated into 2 subclusters. One of them included all other radiated treatments and 100 mM NaCl treatment. On the other subcluster control present in sub-subcluster alone and 50mM NaCl and all putrescine treatments are presents in other sub-subcluster. This pattern of clustering proved the genetic variability of 10Gy+100mMNaCl genotype from control and other treatments.

Principle component analysis (PCA) follows the same pattern of UPGMA dendrogram and demonstrated the genetic diversity of 10Gy+100mMNaCl genotype (Fig 8B).

DISCUSSION

Fabaceous plants are a good answer for expanding populations, improving human and animal food, and enhancing soil fertility (Castro-Guerrero et al. 2016).

Present results show that growth parameters such as stem length, root length, number of leaves, shoot fresh

Table 3. Specific positive amplicons of ISSR and SCoT loci for osmotic stressed *V. faba* treated by gamma radiation and putrescine.

Analysis	Treatment	Primer	Molecular size of positive marker amplicon (bp)
ISSR	50mM NaCl	UBC-823	1600
	100mM NaCl	UBC810	950
		UBC818	925
		UBC849	470
	5mMPut+100mMNaCl	UBC810	170
		UBC-817	1270,1055
	10Gy+50mM NaCl	UBC849	390,260
	10Gy+100mM NaCl	UBC810	641
		UBC818	188
		UBC-823	920
		UBC-817	1455
SCoT	5mMPutrescine	SCoT -52	1600
	10Gy	SCoT -52	1430
	10Gy+50mM NaCl	SCoT -13	2090,885,515
		SCoT -52	1294
	10Gy+100mM NaCl	SCoT -14	712

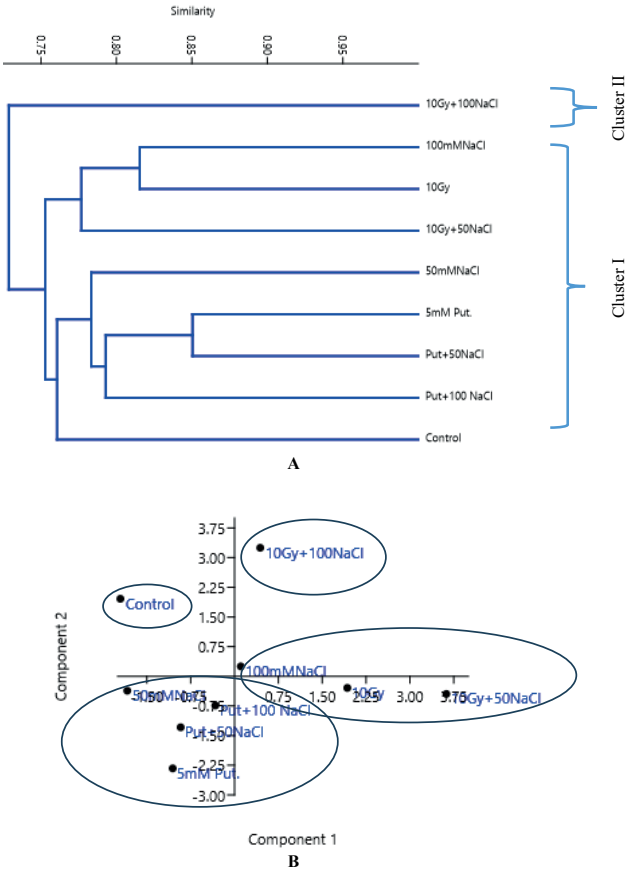


Figure 8. A) UPGMA dendrogram based on ISSR and SCoT markers data. B) Scatter plot of PCA using ISSR and SCoT markers.

weight, shoot dry weight, root fresh weight and root dry weight were decreased by salinity stress. These findings concur with those made on several crops by Sadak and Abd Elhamid (2013).

Putrescine application or gamma radiation treatment of plants were successful in accelerating development in saline environments. This is in line with the findings of Zhao and Qin (2004), who discovered that exogenous application of putrescine improved root development in barley seedlings under salt stress. Furthermore, Khosroshahi and Ashari (2008) demonstrated that strawberry, apricot, peach, and sweet cherry fruits' soluble solids content, weight loss, and titratable acidity were all improved by the foliar application of polyamines, such as putrescine. Many physiological processes and plant response to biotic and abiotic stresses are regulated by substances like putrescine in plants (Alcázar et al. 2010). As they activate or inhibit vegetative growth, blooming, fruiting (Harez and Abbas 2015).

The efficiency of metabolic processes in plants can be increased by polyamine compounds, including putrescine. Additionally, the physiological functions of plants are improved because of the roots' increased ability to absorb nutrients from the soil. These present favorable results of putrescine can be explained by these two factors (Youssef et al. 2007). Moreover, putrescine can accelerate growth, cell division and elongation (El-Bassiouny and Mostafa 2008) by boosting the levels of endogenous growth regulators including auxins, cytokinin, and gibberellins while lowering the efficiency and quantity of growth inhibitors like abscisic acid (ABA).

A form of electromagnetic wave called gamma irradiation has a good effect on molecular penetration and can ionize materials by igniting their electrons (UNSC 2000). Ionized cells might be distinguished by the disruption of host DNA that resulted in noticeable changes in hereditary features. Because DNA has the capacity to repair itself after damage due to the mechanism of proofreading, the perturbation of DNA might be transitory rather than permanent, which would have less severe effects (Ali et al. 2016). Low levels of gamma radiation may have physiological effects on plant growth due to an interaction between gamma rays and cell-based compounds that results in the production of free radicals. According to, the effect of low doses can be summed up by the acceleration of cell division, cell expansion, enzyme activity, tolerance against biotic or abiotic stress, and the increase in plant yield. The

resulted free radicals can alter the primary components of cells (El-Beltagi et al. 2011). According to Aly et al. (2019), utilizing gamma rays increased all growth metrics in eggplant. Low doses of gamma radiation increase plant height which may be related to the ability of radiation to stimulate cell division and other crucial processes that improve nucleic acid synthesis and activation of RNA or protein synthesis (Asare et al. 2017).

Regarding the impact of salinity on the chemical components of the *V. faba* plant, rising salinity level resulted in a decrease in total soluble sugar. Salinity may inhibit photosynthesis activity and/or increase consumption of carbohydrates, according to the reduction in leaf photosynthetic pigments (Hassanein et al., 2009). Additionally, one of the alterations brought by salt and drought stress in plants is proline buildup, which is frequently thought to be implicated in stress resistance processes. The stabilization of proteins and membranes against the denaturation impact of excessive concentrations of salts and other damaging solutes may play a role in maintaining the structure of macromolecules and other organelles (Munns, 2002). With rising salinity levels, salt stress caused declines in total nitrogen and increment in proline and free amino acids (Abdelhamid et al. 2013). These results are very similar to those found by Taie et al. (2013). The decrease in protein synthesis and/or the rise in its breakdown can be linked to the reduction in total nitrogen.

Strong osmo-protectants known as compatible osmolytes (a group of tiny molecules that includes polyamines, glycinebetaine and TSS) help to mitigate the negative consequences of osmotic stress. Putrescine foliar spraying treatment resulted in a significant increase in the percentage of total carbohydrates and nitrogen. This increase in growth may be attributable to this substance's ability to stimulate physiological processes that were improved vegetative growth (El-Bassiouny and Mostafa, 2008). Additionally, putrescine increased the levels of proline, soluble sugars, and amino acids in wheat plants (Hussein et al. 2023). Moreover, putrescine's protective action on wheat's Rubisco protein may be the cause of variance in protein expression in plants developing under water deficiency stress (Hassan et al., 2020). Also, radiation offers protection from the effects of salt stress.

One of the key defensive responses of plant cells to gamma irradiation stress is the development of defense systems (Jan et al., 2012). The increase in the content of soluble protein is one of the plant's defenses against gamma irradiation damages, Hanafy and Ageeb (2018) discovered that leaves produced from irradiated seedlings with lower doses of gamma rays have higher total protein and proline levels. Additionally, Afrin et al. (2019) discovered that onion bulbs with low gamma ray doses had the highest nitrogen content.

According to Jităreanu et al. (2013), the mitotic index is the significant biological parameter that indicates the frequency of cell division and meristem growth. The current findings showed that the negative influence of NaCl on mitotic division of tested *V. faba* meristematic, which was directly related to the salt concentration. This mitodepressive effect on MI was accompanied by an increase in chromosomal abnormalities and the induction of micronucleus rate (Souguir et al. 2022). Mitotic depression may be due to decrease in cyclin dependent kinases (cdcs) activity (Zhao et al. 2014), accumulation of cells at G_1 phase inhibiting DNA synthesis or arresting the cell in G_2, hindering the cell to enter M phase (Mahfouz and Rayan 2017). Foliar application of putrescine and irradiation with gamma radiation mitigated the harmful effect of NaCl causing significant increase in MI frequencies with low mitotic abnormalities percentage as demonstrated before. Previously treatment with putrescine showed enhancement in MI after 6h that supports that polyamine is essential constituents of the cell and implicates in cell growth and proliferation (Gömürgen et al. 2005). El-Azab et al. (2018) proved that exposure to low levels of gamma radiation may stimulate ROS at a very low rate, which can speed up the passage of the cell cycle from G0 to G1, activating plant cell cycle machinery.

The frequencies of the various division stages changed because of treatment with NaCl. Following salt stress, prophase frequency increased considerably, whereas gamma radiation exposure significantly increased cell accumulation during metaphase. Putrescine caused non-significant changes in frequencies of the different phases. These variations in the frequencies of mitosis phases show that NaCl and gamma radiation affects the relative length of each phase of division as compared with the control. The prophase accumulation proves the toxic strength of a treatment caused by delaying in breakdown of nuclear membrane or delaying in chromosome compression because of blocking dividing cells at Chfr point that inhibits prophase/metaphase transition (Sobieh et al. 2014). While the accumulation of dividing cells in metaphase may be due to spindle apparatus disruption leading to prevention of metaphase/anaphase transition (Sobieh and Fahmy 2021). Chromosome stickiness was the most frequent form of abnormality seen with NaCl treatments. Chromosome stickiness indicates that NaCl has harmful effects that are permanent and cause cell death (Gömürgen et al. 2005).

Stickiness may be due to changes in nucleosomes formation and/or absent of specific non-histone proteins implied in chromosome organization essential for chromosome segregation and chromatid separation (Potapova and Gorbsky 2017). Chromosome bridge/or multibridge are developed due to strong adhesion between chromatin fibers which stick sister chromatids at metaphase and hold them together, this strong connection may prevent the correct separation of joined chromatids during mitosis (Bordin et al. 2023). The formation of spindle disturbance and multipolarity by gamma radiation may be resulted from inhibition of spindle fiber formation followed by the random distribution of the chromosomes in the cytoplasm (Singh and Roy, 2017). Moreover, the presence of ring chromosome could be result from loss of telomeric part of chromosome (Khanna and Sharma 2013). The induction of laggards indicates the clastogenic effect of any treatment causing loss of some genetic material (Sobieh et al. 2016). Laggards usually form micronuclei (El-Azab et al. 2018). As well, micronuclei can be formed when lagging free chromosomes cannot reach to the cell poles in the correct time to be included in the major nucleus (Utsunomiya et al., 2002). Micronuclei often serve as a marker of chromosomal instability or reflect sensitivity due to single gene polymorphisms as suggested by Luzhna et al. (2013). Direct relationship for MN induction and salt ions shows the mitodepressive effects of higher salt concentrations. It is also an indicator of cell division sensitivity to ion level, which may interfere with the cell cycle regulatory machines. Cl^- ions can induce variation of genome arrangements and mutations (Boyko et al. 2010) The highly positive relationship between gamma radiation and induction of MN could be due to that gamma radiation can produce chromosomal breaks in two chromosomes that tend to reunite forming a MN (Pampalona et al. 2016).

Many checkpoints control the cell cycle of eukaryotic cells. Before entering mitosis, cell cycle checkpoints measure the size of the cell, ensure accurate chromosome replication, and ensure chromosome integrity. The metaphase checkpoint then starts the correct segregation during mitosis through the mitotic spindle. The reversible phosphorylation of the regulatory protein cyclins is necessary for the cell cycle checkpoint. the activity Cdcs family mediates this phosphorylation (Fouad and Hafez 2018). Therefore, Cdcs are regulatory proteins that regulate transcription and restrict cell division in response to undesirable conditions (Ding et al. 2020). Cdcs are divided into eight types based on the putative cyclin-binding domains. One of them, CDCA, is encoded in *V. Faba* by the *cdc2* gene (Binarova et al. 1998). It has previously been demonstrated that CDCAs are involved in both G1/S and G2/M transitions (Hemerly et al. 1995). To promote MI in *Arabidopsis, cdc2* expression is upregulated prior to cell division (Hemerly et al. 1993). The reduction of salt's influence on the cdcs activities and consequently mitotic division was verified by the current data, where there was a considerable downregulation in cdc2 expression level after salt stress. Reduction in Cdcs and cyclin activity under salt stress was previously reported by Qi and Zhang (2020). In general, the downregulation of *cdc* genes expression is a stress response (Kitsios and Doonan 2011) leading to cell cycle arrest, prolonged S-phase progression, or delayed entry into mitosis (De Veylder *et al*. 2007). Previously, treatment of *V. faba* root tips with bohemine or roscovitine (inhibitors of *cdc2-k* gene) led to characteristic abnormalities in mitosis leading to prolonged prophase with intact nuclear envelope (Binarova et al. 1998).

On the contrary, the alleviation effect of both putrescine and gamma radiation caused highly significant upregulation in mitotic division rate alongside with *cdc2* expression level. The low concentration of NaCl with both putrescine and gamma radiation caused upregulation of the expression level for 2 and 4-folds respectively. This upregulation of *cdc2* expression level accompanied with increase in mitotic division is like the results of Fouad and Hafez (2018), who found increase in mitotic division associated with increase in *cdc2* expression level in *Allim cepa*.

Two fingerprint markers ISSR and SCoT were used to evaluate the genetic diversity among the eight treatment groups of *V. faba* and control. Present data show that ISSR and SCoT molecular markers have produced different patterns of DNA polymorphism and discriminated molecular variations among treated and control *V. faba*. This reveals the incidence of modifications at the molecular level among *V. faba* plants by different treatment used. Afiah et al. (2016) proved that ISSR was beneficial in realizing the genetic variation among *V. faba* genotypes and *V. faba* genotypes under salt stress respectively. The SCoT molecular marker technique is effective in assessing genetic variations between control and stress treated *V. faba* (Essa et al. 2023) and between control and mutants *V. faba* caused by gamma irradiation. These variations caused changes at the molecular and phenotypic levels. In *Vigna unguiculata* the appearance and the disappearance of amplicons in the mutant genotypes compared to control proved the impact of gamma irradiation on phenotypic and genotypic traits (Vanmathi et al. 2021). Moreover, gamma irradiation had the capabilities of induction genetic variation in the genotypes of Cowpea varieties, as assessed by RAPD and ISSR markers (Badr et al. 2014). In the same concern ISSR fingerprints differentiate the amaranth mutant from Ficha cultivar and K-433 hybrid and

showed that the genetic diversity produced may be part of the complex response to the gamma-radiance (Žiarovská et al 2013). Besides, modifications in control and gamma radiated treatments might be produced by inter microsatellite length polymorphism (Aly et al. 2019).

A comparison of the level of polymorphism and discriminating efficacy of ISSR and SCoT showed that each of the two techniques can detect genetic variation among control and treated *V. faba*. But the results revealed that the ISSR markers were more efficient than SCoT for differentiating the genetic variation. ISSR markers effectively assessed the genetic diversity among the *V. faba* and produced a wide range of PIC and medium RP value, which indicates the presence of specific alleles in some genotypes, which can assist the differentiation of these genotypes from the others (Serry et al. 2019). PIC and Rp are reported to be better informative factors than MI to describe the discriminative power of a primer to distinguish various genotypes (Shingote et al. 2019(. Although ISSR was more efficient, both two markers produced high level of polymorphism. Therefore, a combination of ISSR (spanning selected repetitive sequences) and SCoT (targeting the start codon sequences of the DNA) can be considered as best markers for more expressive and dependable investigation of genetic variability as clarified by cluster analysis using UPGMA dendrogram and PCA scatter plot. They revealed high genetic variability of 10Gy+100mM NaCl. So, it can be concluded that gamma radiation was an effective means for initiation mutation in *V. faba*. These changes could be successfully identified by ISSR and SCoT analysis. Therefore, the recent progress in mutation breeding studies in relation with new technologies is quite critical to influence and improve plant breeding programs to overcome the climate changes.

CONCLUSION

Salinity is a severe problem, which affect plant growth and productivity. Plants have developed highly advanced stress tolerance mechanisms to adapt stresses. The application of putrescine or irradiated plants with gamma radiation were effective in enhancing growth, cell division under saline conditions and alleviating the harmful effect of salinity.

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REFERENCES

Abdelhamid MT, Sadak MSH, Schmidhalter URS, EL-Saady M. 2013. Interactive effects of salinity stress and nicotinamide on physiological and biochemical parameters on faba bean plant. Acta biol. Colomb. 18(3):499-510.

Afiah SAI,.Abd El- Gawad ZA , Mohamed TH R , Al-Agwany H H.2016. Salt and Water Deficit Tolerance in some *Vicia faba* L. Genotypes in Relation to Pigments ISSR – PCR Markers and Stress Tolerance Indices. Egypt. J. Bot. 56(1):121-144.

Afrin MS, Kabir MA, Alam MS .2019. Effect of gamma radiation on the growth, yield and quality of four onion accessions. J Agric Vet Sci12(8):68-78.

Albrifcany M S, Hajer S, Askander A. 2022. Genetic diversity among some parental varieties of *Vicia faba* L. using ISSR and SCoT molecular marker techniques. J. of Agricult Chemist Biotechnol, Mansoura Univ. 13(12):129-137.

Alcázar R, Altabella T, Marco F, Bortolotti C, Reymond M, Koncz C, Carrasco P, Tiburcio A F, 2010. Polyamines: Molecules with regulatory functions in plant abiotic stress tolerance. Planta 231:1237-1249.

Ali H, Ghori Z, Sheikh S, Gul A .2016. Effects of gamma radiation on crop production. In: Crop production and global environmental issues. Springer, 27-78.

Aly AA, Eliwa NE, Abd El-Megid MH. 2019. Stimulating effect of gamma radiation on some active compounds in eggplant fruits. EJRSA 32(1):61-73.

Aly AA, Eliwa NE, Maraei RW.2019. Physiological and molecular studies on ISSR in two wheat cultivars after exposing to gamma radiation. Science Asia 45(5):436-445.

AOAC. Association of Official Agricultural Chemist. 1984. Official methods of analysis of the association of official analytical chemist. 14 ed. Washington. D.C. USA.

Asare AT, Mensah F, Acheampong S, Asare-Bediako E, Armah J .2017. Effects of gamma irradiation on agromorphological characteristics of okra (Abel- moschus esculentus L. Moench.). Adv Agric 3:1-7.

Badr A, El-Shazly HH, Halawa MM .2014. Cytological effects of gamma radiation and its impact on growth and yield of M1 and M2 plants of cowpea cultivars. Cytologia. 79(2):195-206.

Bates L S, Waldern R P, Teare I D.1973.Rapid determination of free proline for water stress studies determination of free proline for water stress studies. Plant and Soil. 3:205.

Binarova P, Dolez J, Draber P, Heberle-Bors E, Strnad M, szló Bögre L. 1998. Treatment of Vicia faba root

tip cells with specific inhibitors to cyclin-dependent kinases leads to abnormal spindle Formation. The Plant J 16(6):697-707.

Bordin ER, Santos F, Mannes Y, Martins de Freitas A, Ramsdorf WA.2023. Single and mixture cytogenetic effects evaluation of atrazine and glyphosate herbicides at environmentally relevant concentrations on Allium cepa root meristem cells. Ecotoxicol. Environ. Contam.18(1):38-50. https://doi.org/10.5132/eec.2023.01.06

Boyko A, Golubov A, Bilichak A, Kovalchuk I.2010. Chlorine Ions but Not Sodium Ions Alter Genome Stability of Arabidopsis Thaliana. Plant Cell Physiol. 51:1066-1078.

Caplin N, Willey N.2018. Ionizing Radiation, Higher Plants, and Radioprotection: From Acute High Doses to Chronic Low Doses.Front. Plant Sci. 9: 847.

Castro-Guerrero NA, Cui Y, Mendoza-Cozatl D. 2016. Purification of translating ribosomes and associated mRNAs from soybean (Glycine max). Curr. Protoc. Plant Biol 1:1-12. https://doi.org/10.1002/cppb.20011.

Chesnokov Yu V, Artemyeva AM. 2015. Evaluation of the measure of polymorphism information of genetic diversity Agricult. Biol. 50(5):571-578.

Collard B C, Mackill D J.2009. Start codon targeted (SCoT) polymorphism: A simple, novel DNA marker technique for generating gene-targeted markers in plants. Plant Mol. Biol. Rep. 27: 86-93.

De Veylder L, Beeckman T, Inze D. 2007. The ins and outs of the plant cell cycle. Nat. Rev. mol. cell. Biol. 8:655-665.

Demiral T, Türkan ˙I. .2005. Comparative lipid peroxidation, antioxidant defense systems and proline content in roots of two rice cultivars differing in salt tolerance. Environ. Exp. Bot. 53:247-257.

Ding L, Cao J, Lin W, Chen H, Xiong X, Ao H, Yu M, Lin J, Cui Q. 2020.The Roles of Cyclin-Dependent Kinases in Cell-Cycle Progression and Therapeutic Strategies in Human Breast Cancer. Int J Mol Sci. 21:1960.

El-Azab EM, Soliman M, Soliman E, Badr A. 2018. Cytogenetic Impact of Gamma Irradiation and Its Effects on Growth and Yield of Three Soybean Cultivars. Egypt. J. Bot. 58(3):411-422.

El-Bassiouny H M S, Mostafa H A. 2008. Physiological responses of wheat plant to foliar treatments with arginine or putrescine. Austr. J. of Basic Appl Sci. 2(4):1390-1403.

El-Beltagi HS, Ahmed OK, El-Desouky W .2011. Effect of low doses γ-irradiation on oxidative stress and secondary metabolites production of rosemary (*Rosmarinus officicnalis L.*) callus culture. Radiat Phys Chem 80:968-976.

Essa SM, Wafa H A, Mahgoub E.-S.I., Hassanin A A, Al-Khayri J M, Jalal A S, El-Moneim D A, ALshamrani S M, Safhi F A, Eldomiaty. A S.2023. Assessment of eight faba bean (*Vicia faba* L.) cultivars for drought stress tolerance through molecular, morphological, and physiochemical parameters. Sustainab. 15:3291. https://doi.org/10.3390/su15043291

FAO Statistics. 2021. Faosta, Url: http://faostat3.fao.org/download/Q/QC/E

FAOSTAT. 2022. Food and Agriculture Organization of the United Nations. Retrieved from http://www.fao.org/faostat

FAOSTAT 2018. Food and Agriculture Organization of the United Nations. Retrieved from http://www.fao.org/faostat/en/#data/QC

Fouad AS, Hafez RM. 2018. The effects of silver ions and silver nanoparticles on cell division and expression of cdc2 gene in *Allium cepa* root tips. Biol. Plant. 62(1):166-172. https://doi.org/10.1007/s10535-017-0751-6166

Gömürgen A N, Mutlu , Bozcuk S.2005. Effects of Polyamines (Putrescine, Spermidine and Spermine) on Root Tip Mitosis and Chromosomes in *Allium cepa* L Cytologia 70(2):217-224.

Gupta K, Dey A, Gupta B. 2013. Plant polyamines in abiotic stress responses. Acta Physiol Plant. 35(7):2015-2036.

Hammer, Q, Harper D A, Ryan PD, 2001. PAST: Paleontological statistics software package for education and data analysis. Palaeontol. Electron. 4(1):9.

Hanafy RS, Ageeb S. 2018. Physiological and molecular studies on the effect of gamma radiation in fenugreek (*Trigonella foenum* L.) plants. J Genet Eng Biotechnol. 16(2):683-692.

Harez RO, Abbas AJ. 2015. Effect of spraying with organic fertilizer (humic acid) and the Brassinosteroid growth regulator on knots, shedding and some traits of vegetative, flowering and fruiting growth of local orange *Citrus sinensis* L Diyala J Agric Sci. 7(2):78-89.

Hassan N, Ebeed H, Aljaarany A. 2020. Exogenous application of spermine and putrescine mitigate adversities of drought stress in wheat by protecting membranes and chloroplast ultra-structure. Physiol. Mol. Biol. Plants 26:2339245.

Hassanein RA, Bassony FM, Barakat DM, Khalil RR. 2009. Physiological effects of nicotinamide and ascorbic acid on Zea mays plant grown under salinity stress. 1- Changes in growth, some relevant metabolic activities and oxidative defense systems. Res J Agric Biol Sci. 5(1):72-80.

Hemerly A, De Almeida Engler J, Bergounioux C. Montagu M, Engler G, Inzé D, Ferreira P.1995. Dominant

negative mutants of the CDC2 kinase uncouple cell division from iterative plant development. EMBO J. 14:3925-3936.

Hemerly AS, Ferreira P, Engler J, Van Montagu M, Engler G, lnze D.1993. *cdc2a* expression in Arabidopsis is linked with competence for cell division. Plant Cell 5:1711-1723.

Hirt H, Heberle-Bors E. 1994. Cell cycle regulation in higher plants. Sem. Dev. Biol. 5:147-154.

Hoagland DR, Arnon DI. 1950. The water-culture method for growing plants without soil. Circular. Calif. Agric. Exp. Stn. 347:32.

Hussein HA, Alshammari SO, Abd El-Sadek ME, Kenawy SKM. Badawy AA. 2023. The Promotive Effect of Putrescine on Growth, Biochemical Constituents, and Yield of Wheat (Triticum aestivum L.) Plants under Water Stress. Agriculture 13(3):587. https://doi.org/10.3390/agriculture13030587

Isayenkov S, Maathuis FJM. 2019. Plant salinity stress: many unanswered questions remain. Front. Plant. Sci. 10:80. https://doi.org/10.3389/fpls.2019.00080.

Jan S, Parween T, Siddiqi TO. 2012. Effect of gamma radiation on morphological, biochemical, and physiological aspects of plants. Environ. Rev. 20:17e39.

Jităreanu A, Pădureanu S, Tătăringă G, Tuchiluş C, Stănescu U.2013. Evaluation of phytotoxic and mutagenic effects of some cinnamic acid derivatives using the Triticum test. Turk J Biol. 37:748-756.

Khanna N, Sharma S. 2013., "Allium cepa root chromosomal aberration assay: a review," Indian J. Pharm. Biol. Res. 1:105-119.

Khosroshahi MRZ, Ashari EM. 2008. Effect of Putrescine application on post-harvest life and physiology of strawberry, apricot, peach and sweet cherry fruits. J. Agric. Sci. Technol. 45:219-230.

Kitsios G, Doonan JH. 2011. Cyclin dependent protein kinases and stress responses in plants. Plant Signal. Behav. 6:204-209.

Kuznetsov VV, Shevyakova NI. 2007. Polyamines and stress tolerance of plants. Plant Stress. 1:50-71.

Livak KJ, Schmittgen TD. 2001.Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods. 25(4):402-408.

Lowery O H, Rosebrough N J, Farr A L, Ronodall R J.1951. Protein measurement with Folin reagent, J. Biol. Chem. 193(1):265-275.

Ludovici G M, Oliveira de Souza S, Chierici A, Cascone M G, d'Errico F, Malizia A. 2020. Adaptation to Ionizing Radiation of Higher Plants: From Environmental Radioactivity to Chernobyl Disaster. J. Environ. Radioact. 222:106375.

Luzhna L, Kathiria P, Kovalchuk O. 2013. Micronuclei in genotoxicity assessment: From genetics to epigenetics and beyond. Front. Genet. 4:131.

Magyar Z, Mészaros T, Miskolczi P. et al. 1997. Cell cycle phase specificity of putative cyclin-dependent kinase variants in synchronised alfalfa cells. Plant Cell. 9:223-235.

Mahfouz H, Rayan W. 2017. Antimutagenics effects of Stigmasterol on two salt stressed *Lupinus termis* Cultivars Egypt J Genet Cytol. 46:253-272.

Matkovics B, Kecskemeti V, Varga S I, Novak Z, Kertesz Z. 1993. Antioxidant properties of di-and polyamines. Comp Biochem Physiol Comp Biochem. 104:475-479.

Munns R. 2002. Comparative physiology of salt and water stress. Plant Cell Environ. 25(2):230-250.

Nigg E. 1995. Cyclin-dependent kinases: key regulators of the eukaryotic cell cycle. Bioessays. 17: 471-479.

Pampalona J, Roscioli E, Silkworth WT, Bowden B, Genescà A, Tusell L, Cimini D. 2016. Chromosome bridges maintain kinetochore microtubule attachment throughout mitosis and rarely break during anaphase. PLoS ONE 11(1):e0147420.

Potapova T, Gorbsky GJ. 2017. The Consequences of chromosome segregation errors in mitosis and meiosis. Biology 6(1):12.

Qahtan AA, Al-Atar A, Abdel-Salam EM, El-Sheikh MA, Gaafar ARZ, Faisal M. 2021. Genetic diversity and structure analysis of a worldwide collection of faba bean (*Vicia faba*) genotypes using ISSR markers. Int. J. Agric. Biol. 25:683-691.

Qi F, Zhang F. 2020. Cell Cycle Regulation in the Plant Response to Stress. Front. Plant Sci. 10:1765.

Rosen H. 1957. A modified ninhydrin colormetric analysis for amino acid nitrogen. Archives Biochemist Biophys. 67(1):10-15.

Sadak MSh, Abd Elhamid EM. 2013. Physiological response of flax cultivars to the effect of salinity and salicylic acid. J Appl Sci Res. 9(6):3573-3581.

Sequera-Mutiozabal M, Antoniou C, Tiburcio AF, Alcázar R, Fotopoulos V. 2017. Polyamines: emerging hubs promoting drought and salt stress tolerance in plants. Curr Mol Biol Rep. 3:28-36.

Serry N, Ali KH, Hussein MSM. 2019. Phenotypic, Biochemical and Molecular Characterization of New Egyptian Mango Genotypes. Hortsci J Suez Canal Univ. 8(1):55-68.

Shingote PR, Amitha Mithra SV, Sharma P, Devanna NB, Arora K, Holkar SK, Khan S, Singh J, Kumar S, Sharma TR, Solanke AU. 2019. LTR retrotransposons and highly informative ISSRs in combination are potential markers for genetic fidelity testing of tissue culture-raised plants in sugarcane. Mol Breed 39(2):25.

Singh D, Roy BK. 2017. Evaluation of malathion-induced cytogenetical effects and oxidative stress in plants using Allium test. Acta Physiol. Plant. 39(4):92.

Snedecor GW, Cochran WG. 1980. Statistical methods, Lowa State University Press/Ames. pp. 177-195.

Sobieh Sh S, Fahmy DM. 2021. Assessment of antimitotic and programmed cell death stimulation potentials of *Galium sinaicum* (Delile ex Decne) Boiss. at the cellular level. Caryol 74(2):21-35. https://doi.org/10.36253/ caryologia-1057

Sobieh Sh S, Tawab SA, Fahmy DM. 2014. Deregulation of mitosis progression and cytotoxic effect triggered in *Allium cepa* L. Roots by *Rubus sancatus* Schreber Extract. Life Sci J 11:1047-1058.

Sobieh Sh S, Zeinab MHKh, Abeer AR, Naglaa ANY. 2016. In vitro and in vivo genotoxicity and molecular response of silver nanoparticles on different biological model systems. Caryol 69:147-161.

Souguir D, Abd-Alla HI, Hörmann G, Hachicha M. 2018. Chromosomal and nuclear alterations in the root tip cells of *Vicia faba* induced by sodium chloride. Water Environ Res. 90:164-171.

Souguir D, Berndtsson R, Mzahma S, Filali H, Hachicha M. 2022. Vicia micronucleus test application for saline irrigation water risk assessment. Plants 11:462. https://doi.org/10.3390/ plants11030462

Spencer LMM, Forster BP, Jankuloski L. 2018. Manual on mutation breeding, 3rd ed.; Food and Agriculture Organization of the United Nations (FAO): Rome, Italy.

Taie H, Abdelhamid MT, Dawood MG, Nassar RM. 2013. Presowing seed treatment with proline improves some physiological, biochemical and anatomical attributes of faba bean plants under sea water stress. J Appl Sci Res. 9(4):2853-2867.

UNSC .2000. Sources and effects of ionizing radiation: sources (Vol. 1). United Nations Publications.

Utsunomiya KS, Bione NCP, Pagliarini MS. 2002. How many different kinds of abnormalities could be found in unique endogamous maize plant? Cytologia, 67:169-176.

Vanmathi S, Arulbalachandran D, Soundarya V. 2021. Effects of gamma radiation on quantitative traits and genetic variation of three successive generations of cowpea (Vigna unguiculata (L.) Walp.) Plant Sci. Today, 2021 8(3):578-589. https://doi.org/10.14719/ pst.2021.8.3.1054

Velikova V, Yordanov I., Edreva A. 2000. Oxidative stress and some antioxidant systems in acid rain-treated bean plants: Protective role of exogenous polyamines. Plant Sci. 151:59-66.

Venkatesan J, Ramu V, Sethuraman Th, Sivagnanam Ch, Doss G. 2021. Molecular marker for characterization of traditional and hybrid derivatives of *Eleusine coracana* (L.) using ISSR marker. J Genet. Eng. Biotechnol. 19:178.

Wild SA, Corey RB, Lyer JG, Voigt GK. 1985. Soil and plant analysis for tree culture. Oxford and IPH Publishing Co., New Delhi, India, pp. 529-546.

Wisniewski M, Nassuth A, Teulieres CH, Marque CH, Rowland J, Cao PHB. 2014. Genomics of cold hardiness in woody plants. Crit Rev Plant Sci. 33:92-124.

Xavier MN, Torres Novaes JA, Cavalcante Silva AC, Alves AVS, Araujo Santos MJ, Pantale˜ao SM, Scher R, d'Errico F, Oliveira de Souza S. 2023. Cytogenetic effects of β-particles in *Allium cepa* cells used as a biological indicator for radiation damages. J Environ Radioact. 259:107-109.

Xiong F, Zhong R, Han Z, Jiang J, He L, Zhuang W, Tang R. 2011. Start codon targeted polymorphism for evaluation of functional genetic variation and relationships in cultivated peanut (*Arachis hypogaea* L.) genotypes. Mol Biol Rep 38:3487-3494.

Youssef EAE. 2007. Increasing drought tolerance of gladiolus plants through application of sustainable agricultural development. Agadir, Morocco, pp. 299-326.

Zhang XZ. 1993. Crop physiology research method. China Agricultural Press, Beijing, pp. 145-146.

Zhao F, Qin P. 2004. Protective effect of exogenous polyamines on root tonoplast function against salt stress in barley seedlings. Plant Growth Regul. 42:97-103.

Zhao L, Wang P, Hou H, Zhang H, Wang Y, Yan S, Huang Y, Li H, Tan J, Hu A, Gao F, Zhang Q, Li Y, Zhou H, Zhang W, Li L. 2014. Transcriptional regulation of cell cycle genes in response to abiotic stresses correlates with dynamic changes in histone modifications in maize. PLoS One 9:e106070

Žiarovská J, Ražná K, Labajová, M. 2013. Using of inter microsatellite polymorphism to evaluate gamma-irradiated amaranth mutants. Emir J Food Agric 25(9):673-681.

Ziêtkiewicz E, Rafalski A, Labuda D.1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics. 20:176-183.

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Karyological studies of four species of Lady's Slipper Orchids (*Paphiopedilum*) collected in the Bogor Botanical Garden, Indonesia

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Abstract. *Paphiopedilum* is one of the most widely grown and hybridized of all orchid genera due to its distinctive flower morphology. This genus consists of 139 accepted species and is native to southern China to tropical Asia. Karyological studies on the genus *Paphiopedilum* have been reported by many cytologists in different countries. However, many Indonesian members of *Paphiopedilum* have remained comparatively limited in investigated cytologically. This study aimed to analyze karyological characters of four species of *Paphiopedilum* collected in the Bogor Botanical Garden, Indonesia, namely *P. armeniacum*, *P. hirsutissimum, P. primulinum,* and *P. superbiens*. Karyological studies were conducted by root tips squash method. The results showed that four species of *Paphiopedilum* have a basic chromosome number of x = 15. They are diploid with 2n=30, instead of the common diploid chromosome number of *Paphiopedilum* (2n = 26). *Paphiopedilum hirsutissimum* and *P. primulinum* possess 16 m + 14 sm chromosome formulae. Whereas *Paphiopedilum armeniacum* and *P. superbiens* revealed 16 m + 14 sm and 18 m + 12 sm chromosome formulae, respectively. A Robertsonian change in chromosome number generated by the fission of chromosomes would best explain the origin of the new diploid chromosome number and karyotypes of these species.

Keywords: Bogor Botanical Garden, chromosome, diploid, karyotype, *Paphiopedilum.*

INTRODUCTION

Paphiopedilum Pfitzer (Orchidaceae) is known as a lady's slipper orchid and belongs to the subfamily Cypripedioideae Lindley (1840) with other four genera, namely *Cypripedium, Mexipedium, Phragmipedium*, and *Selenipedium*. The subfamily was separated from other subfamilies based on its special characteristic of having two separated fertile anthers (Cribb 1998). *Paphiopedilum* has unique characters that distinguished from genera *Cypripedium* and *Selenipedium*, viz. conduplicate coriaceous leaves, as opposed to the pli-

cate persistent leaves of the latter two genera. *Paphiopedilum* differs from *Phragmipedium* and *Mexipedium*, as they have imbricate sepal vernation, different chromosome base numbers, and a unilocular ovary (Albert and Chase 1992; Albert 1994).

Paphiopedilum is the most popular orchid genus for ornamental plants, because of its distinctive flower morphology (Lee et al. 2011). Therefore, members of this genus are widely grown and hybridized of all orchids (Cribb 1998; Lan and Albert 2011). Ecologically, species of *Paphiopedilum* are important to narrow endemics in various mainland and islands habitat and range from seaside cliffs to montane rainforests (Cribb 1998). Based on the morphological characteristics of inflorescence, leaf type, floral morphology, and molecular data on ITS sequences, Cribb (1997) divided this genus into three subgenera, namely *Parvisepalum*, *Brachypetalum,* and *Paphiopedilum*. After Cribb (1997), several new species and treatments have been described for this genus, therefore this genus consisted of approximately 98 species worldwide by the year 2000 (Koopowitz 2000). The subgenus *Paphiopedilum* is classified into five sections, viz. *Coryopedilum*, *Pardalopetalum*, *Cochlopetalum*, *Paphiopedillum*, and *Barbata* (Lee et al. 2017; Tsai et al. 2020) based on morphological, cytological and molecular data (Cox et al. 1997; Cribb 1998; Chochai et al. 2012).

Paphiopedilum is distributed in four of 25 biodiversity hotspots (Myers et al. 2000), the Indo-Burma, Sundaland, Wallacea, and Philippines, which are also the "major evolutionary hotspots" (de Bruyn et al. 2014). According to Tsai et al. (2020), phylogeny and historical biogeography of *Paphiopedilum* reveals the relevance of the differentiation of *Paphiopedilum* in Southeast Asia and geological history. The mountain forests of Indonesia are home to great diversity of the endemic *Paphiopedilum* species. It is predicted that at least 50% of the Lady's Slipper Orchids of the world can be found naturally in the mountains forest of Indonesia, most of them are endemic to the mainland of Indonesia. For example, *P. agusii* Cavestro & N Bougourd, *P. anitanum* Cavestro, *P. braemii* H.Mohr ., *P. dodyanum* Cavestro, *P. glaucophyllum* J.J. Smith, *P. intaniae* Cavestro, and *P. javanicum* (Reinw. ex Lindl.) Pfitzer are endemic to Java (Govaerts et al. 2018).

Chromosome number and karyomorphological analysis is very significant for understanding the genome structure, its organization, and evolution within a genus of certain plant taxon at inter- and intra-specific levels (Ehrendorfer 1980). Analyses of chromosome numbers are very important, because they represent a fundamental step in the study of any taxa of organisms. Chromosome counts provide indispensable information on genetic discontinuities within and among species, and they contribute to the understanding of phylogenetic relationships at all taxonomic levels (Windham and Yatskievych 2003).

Extensive chromosome account and karyotype analysis of the Lady's Slipper Orchids have been published. Chromosome counts are published for almost all species of *Paphiopedilum* and the detailed karyotypes are also available for most species of the genus (Kamemoto et al. 1963; Karasawa 1979; Karasawa and Aoyama 1980; Karasawa 1982, 1986; Cox et al. 1998). However, chromosome account and karyotype analysis for Indonesian *Paphiopedilum* are scanty although most of species of this genus are distributed in Indonesia. This study aimed to observe chromosome numbers and conduct the karyotipe analysis of some species of Indonesian *Paphiopedilum*. Accumulation of cytological data would be very important in providing reference to breeding programs.

MATERIALS AND METHODS

Plant materials

Root tips of four species of *Paphiopedilum* in various subgenera were taken from the orchids in vitro culture collection of the Plant Tissue Culture Laboratory and the orchid plants collection of the Bogor Botanical Garden (BBG). *Paphiopedilum primulinum, P. superbiens* were collected from the wild population, whereas *P. armeniacum* and *P. hirsutissimum* were introduced plants (Table 1).

Somatic chromosome observations

Procedures for somatic chromosome observation followed by Manton (1950) and modified by Praptosuwiryo and Darnaedi (2008). The actively growing roots were used for chromosome preparation. Root tips pretreated with 0.001 M 8-hydroxyquinolin at 4°C for 24-26 hours. The root tips then were fixed in 45% acetic acid for 10 minutes at room temperature after being rinsed with distilled water. Root tips were macerated with 45% acetic acid (CH_3COOH): 1N HCl (1:3) at 60°C for 4 minutes, and then stained in 1% aceto-orcein. The meristematic cells were squashed in a drop of 1% acetic acid orcein under a coverslip of 22 x 22 mm on a microscope slide. Chromosome observation was performed under the microscope using a 100x magnification objective with the addition of immersion oil. An Olympus micro-

Table 1. Living material for karyological studies of the genus *Paphiopedilum* collected in the Bogor Botanical Garden, Indonesia.

No.	Species	Subgenus/Section	Distribution	Status
1.	*P. armeniacum* S.C.Chen & F.Y.Liu	*Parvisepalum/ Parvisepalum*	China	introduced
2.	*P. hirsutissimum (*Lindl. ex Hook.) Stein	*Paphiopedilum /Paphiopedilum*	China, India, Lao PDR, Myanmar, Thailand, Vietnam	introduced
3.	*P. primulinum* M.W.Wood & P.Taylor	*Paphiopedilum/Cochlopetalum*	Sumatra	wild, collected from Aceh
4.	*P. superbiens* (Rchb.f.) Stein	*Paphiopedilum /Barbata*	Sumatra	wild, collected from North Sumatra

Table 2. Chromosome shape classification based on long and short arms ratio.

Arm Ratio (AR)	Sentromer position	Chromosome shape
1.0 ≤ AR < 1.7	median	metasentric (m)
1.7 ≤ AR < 3.0	submedian	submetasentric (sm)
3.0 ≤ AR < 7.0	subterminal	subtelosentric (st)
7.0 ≤ AR < ∞	near terminal	acrosentric (a)
∞	terminal	telosentric (t)

scope U-TV0 with the objective 100x connected to a digital camera (5XC-3 5H12344) with a computer monitor was used to capture the images of well-spread chromosome complements.

Data analysis

The long-arm and the short-arm length of each chromosome were recorded using 5 μm as the unit. Each chromosome picture was cut out and arranged in descending order of length. Karyotype analysis was based on mitotic metaphase cells from each species. Chromosome shape at metaphase was classified based on arm ratio (AR) (Levan et al. 1964) (Table 2). Chromosome characteristics were measured using Ideokar 1.2 software (Mirzaghaderia and Marzangib 2015).

RESULTS

In the present investigation, the numbers of somatic chromosomes of four species of *Paphiopedilum* were counted and presented in Table 3 and Figures 1.

The four species of *Paphiopedilum* disclosed the same chromosome number of 2n=30. Somatic chromosomes at metaphase and the idiogram of four species of *Paphiopedilum* are shown in Figure 1. The chromosome shapes of *P. armeniacum*, *P. hirsutissimum*, *P. primulinum*, and *P. superbiens* are 16 m + 14 sm, 24 m + 6 sm, 24 m + 6 sm, and 18 m + 12 sm, respectively (Table 3.).

DISCUSSION

Paphiopedilum possesses unusually large chromosomes for orchids (Kamemoto et al. 1963). The basic cytology of *Paphiopedilum* is reasonably well studied and chromosome numbers have been published for many species (Karasawa 1979, 1986; Karasawa and Aoyama 1980, 1988; Karasawa and Tanaka 1980, 1981; Karasawa and Saito 1982; Cox et al. 1998). However, almost all species reported were outsite of Indonesia.

Four species of *Paphiopedilum* cultivated in the Bogor Botanical Garden have chromosome number of 2n = 30 (Figure 1). Chromosome numbers of the four species were reported for the first time. Karyological studies of *Paphiopedilum* have shown considerable chromosomal variation, which ranges from 2n = 26 to 2n =

Table 3. Karyotypic characters of four species of *Paphiopedilum* collected in the Bogor Botanical Garden.

No.	Species	Chromosome					
		Chromosome number (2n)	Long arm (μm)	Short arm (μm)	Total arm Length (μm)	Arm ratio (μm)	Chromosome shape
1.	*P. armeniacum* S.C.Chen & F.Y.Liu	30	3.23–5.24	1.12–4.60	4.32–9.80	1.13–1.25	16 m + 14 sm
2.	*P. hirsutissimum (*Lindl. ex Hook.) Stein	30	2.36–5.73	1.21–4.33	3.58–10.05	1.14–2.03	24 m + 6 sm
3.	*P. primulinum* M.W.Wood & P.Taylor	30	1.58–7.07	1.21–5.31	3.00–12.38	1.11–1.76	24 m + 6 sm
4.	*P. superbiens* (Rchb.f.) Stein	30	2.36–5.73	1.21–4.33	3.58–10.05	1.27–1.95	18 m + 12 sm

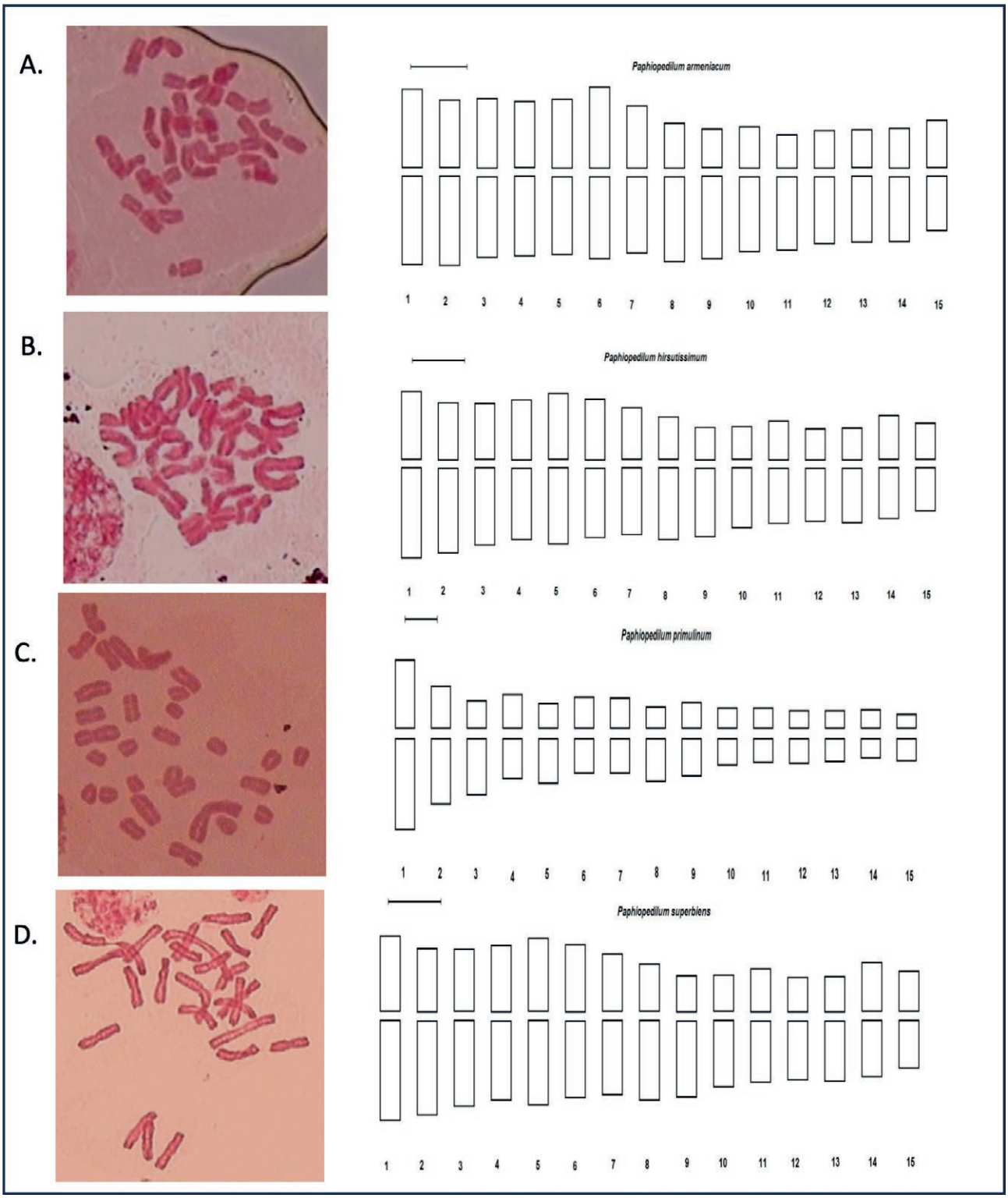

Figure 1. Somatic and ideogram metaphase chromosomes of *Paphiopedilum*, Ideogram of each species is on the right side of the image: (A) *P. armeniacum* (2n = 30), (B) *P. hirsutissimum* (2n = 30), (C) *P. primulinum* (2n = 30), (D) *P. superbiens* (2n = 30). Scale bars: 5 μm.

42 (Karasawa 1979). However, species of *Paphiopedilum* have the multiple chromosomes of the basic chromosome number x = 13 as reported by Lee et al. (2017). Species in the sections *Barbata* and *Cochlopetalum* of subgenus *Paphiopedilum* have a variety of chromosome numbers ranging from 2n = 28 to 42 and 2n = 30 to 37, respectively (Cox et al. 1998).

Paphiopedilum armeniacum S.C.Chen & F.Y.Liu

Paphiopedilum armeniacum belongs to subgenus *Parvisepalu*m, section *Parvisepalu*m (Lan and Albert 2011). This species is currently listed as endangered in The IUCN Red List of Threatened Species, version 2014.3 and grows in the restricted area to a river valley in Yunnan, China on limestone substrates in rocky and brushy habitat (Rankou and Averyanov 2015).

The chromosome account of *P. armeniacum* cultivated in the Bogor Botanical Garden with 2n = 30 is a new record for chromosome number for this species. Lee et al. (2018) reported a cytotype of *P. armeniacum* with 2n = 26 from Taiwan. This species is usually reported to have chromosome 2n = 26 (Lan and Albert 2011).

The new karyotype of *P. armeniacum*, with 2n = 30, with the centromeric formula of chromosomes 16 m + 14 sm reported here (Figure 1A), expands the chromosome number range for subgenus *Parvisepalum*, which was previously considered to be conserved at 2n = 26 (Chochai et al. 2012). The members of subgenus *Parvisepalum* usually show 2n = 26m (Lee et al. 2011).

Paphiopedilum hirsutissimum (Lindl. ex Hook.) Stein.

Paphiopedilum hirsutissimum is a member of subgenus *Paphiopedilum,* section *Paphiopedilum* (Lan and Albert 2011). This species is distributed in northern and western Guangxi, southern and western Guizhou, southern and eastern Guizhou, northern and eastern India, Laos, Thailand, and northern Vietnam (Liu et al. 2009; Li et al. 2015; Chen et al. 2018). This species usually grows on the crevices on shaded cliffs or rocky and well-drained places in forests or thickets in limestone areas at the elevation of 700–1500 m asl.

Chromosome number of *P. hirsutissimum* cultivated in the Bogor Botanical Garden reported here is 2n = 30. Formerly cytological records showed that most species of the subgenus *Paphiopedilum*, section *Paphiopedilum*, have diploid chromosome number 2n = 26 (*P. fairrieanum, P. henryanum, P. hirsutissimum, P. tigrinum*), except *P. druyi* (2n = 30) (Lan and Albert 2011).

The chromosome formula of *P. hirsutissimum* reported here is 2n = 24 m + 6 sm (Figure 1B). Chromosome formula in the subgenus *Paphiopedilum* are vary, for example, 2n = 26 m of *P. rothschildianum* (section *Coryopedilum*), 2n = 20m + 12t of *P. callosum* (section *Barbata*), 2n = 14m + 22t of *P. glaucophyllum* (section *Cochlopetalum*) (Lee et al. 2011).

Paphiopedilum primulinum M.W.Wood & P.Taylor.

Paphiopedilum primulinum is endemic to Sumatra (southern Aceh). It is a member of section *Cochlopetalum* and closely related to *P. liemianum* based the molecular data, such as nuclear ribosomal ITS, plastid *trnL* intron, *trnL-F* spacer, and *atpB-rbcL* spacer (Tsai et al. 2020).

The chromosome account of *P. prmulinum* with 2n = 30 is reported here for the first time. Formerly cytological study *P. primulinum* of Sumatra has been reported to have 2n = 32 (Lan and Albert 2011). The chromosome formula for *P. primulinum* reported here is 2n = 24 m + 6 sm (Figure 1C).

Paphiopedilum superbiens (Rchb.f.) Stein.

Paphiopedilum superbiens belongs to subgenus *Sigmatopetalum,* section *Barbata* (Karasawa and Saito 1982). According to Braem and Chiron (2003), this species is included in the *Ciliolare* complex of subsection *Loripetalum*. Based on the phylogenetic relationships resulting from analysis of the combined molecular data matrix, such as nuclear ribosomal ITS, plastid *trnL* intron, *trnL-F* spacer, and *atpB-rbcL* spacer, *P. superbiens* is closely related to *P. curtisii* and come into section *Barbata* (Tsai et al. 2020).

The chromosome account of *P. superbiens* with 2n = 30 is reported for the first time. A formerly cytological study of the members of the section *Barbata* revealed 2n = 36 for *P. curtisii* and *P. dayanum*, 2n = 38 for *P. acmodontum* and *P. sangii,* 2n = 40 for *P. sukhakulii* and *P. venustum* (Lan and Albert 2011). The chromosome formula for *P. superbiens* reported here is 2n = 18 m + 12 sm (Figure 1D).

Paphiopedilum (Orchidaceae: Cypripedioideae) is characterized by considerable chromosome number variation (2n = 26–42). Chromosome numbers vary from 2n = 28–42 and 2n = 30–37 in the sections *Barbata* and *Cochlopetalum* of subgenus *Paphiopedilum*, respectively (Lee et al. 2018). According to Cox et al. (1998), the most common diploid chromosome number in *Paphiopedilum* is 26 metacentric chromosomes, as displayed in subgenus *Parvisepalum, Brachypetalum* and *Paphiopedilum*, mainly sections *Pardalopetalum*, *Coryopedilum*, and *Paphiopedilum*. However, the chromosome number 2n = 30 was also reported. This diploid chromosome number occured in *P. druryi* and *P. spicerianum* (Cox et al. 1998). The chromosome account of *Paphiopedilum* obtained from this current study supported the existence of 2n = 30 for the genus. Prior to this study, 2n = 30 was

reported for the hybrid species of *P. rothschildianum* × *P. moquetteanum* (Lee et al. 2011).

The changes in chromosome number and karyotype symmetry were considered as a consequence of Robertsonian centric fission (Jones 1998). A Robertsonian relationship between the different karyotypes whereby changes in chromosome number are generated by the fission or fusion of chromosomes at or near the centromere to generate either telocentric or metacentric chromosomes, respectively (Robertson 1916). Duncan and MacLeod (1949, 1950) proposed Robertsonian change to explain the maintenance of arm number in the two genera of Orchidaceae, namely *Paphiopedilum* and *Phragmipedium*. Cox et al. (1997) postulated centric fission of metacentric chromosomes into telocentrics as the predominant mechanism of karyotype evolution in *Phragmipedium* and *Paphiopedilum* and that karyotype orthoselection is operating in some species groups. *Paphiopedilum* species display many chromosomal rearrangements like duplications, translocations, and inversions. Therefore the genus is a good model system for the study of complex chromosomal evolution in plants (Lan and Albert 2011).

The phylogeny of *Paphiopedilum* has been reported by Chochai et al. (2012) and Tsai et al. (2020) using molecular data (nuclear and plastid DNA) which each species studied here are separated in different clades both subgenus and section levels. However, their chromosome shape does not correlate with clade separation, mainly in subgenus *Paphiopedilum*. *P. hirsutissimum* (sect. *Paphiopedilum*) and *P. primulinum* (sect. *Cochlopetalum*) have similar chromosome shape of 24 m + 6 sm, but based on ITS nrDNA and plastid DNA sequence data, they were separated in different clade. It tends that clade separation among subgenus and section within subgenus *Paphiopedilum* are strongly correlated with morphological and molecular data. However, extensive chromosome account and karyotype analysis of *Paphiopedilum* are necessary to construct their relationships and evolution in the future.

CONCLUSION

Chromosome account and the karyotypes of four species of *Paphiopedilum* cultivated in the Bogor Botanical Garden, Indonesia were investigated. New diploid chromosome number was reported. The chromosome number was uniformly 2n = 30. Chromosome account of *Paphiopedilum* used in the study supports the finding that this genus has variations in the number of chromosomes. *P. hirsutissimum* and *P. primulinum* exhibited remarkably similar karyotypes, with centromeric formulae 24 m + 6 sm. Whereas *Paphiopedilum armeniacum* and *P. superbiens* possessed 16 m + 14 sm and 18 m + 12 sm chromosome formulae, respectively. These findings supported the statement that *Paphiopedilum* is a good model system for the study of complex chromosomal evolution in plants.

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AUTHORS' CONTRIBUTIONS

TNgP, RVG, and EH conceived the study. TNgP designed the study; TNgP, JRW, and IAF wrote the manuscript; TNgP, RVG, and EH conducted chromosome observations and studied morphological comparison among species studied; JRW and IAF conducted the karyotypic analysis. All authors read and approved the final manuscript.

REFERENCES

Albert VA, Chase MA. 1992. *Mexipedium*: a new genus of slipper orchid (Cypripedioideae: Orchidaceae). Lindleyana 7: 172–176.

Albert VA. 1994. Cladistic relationships of the slipper orchids (Cypripedioideae: Orchidaceae) from congruent morphological and molecular data. Lindleyana 9: 115–132.

Braem G, Chiron G. 2003. *Paphiopedilum*. Voreppe: Tropicalia.

Chen L, Dong H, Wang JW, Li LN, Xu M. 2018. Microsatellites characteristics analysis and SSR marker development for *Paphiopedilum hirsutissimum* based on transcriptome sequencing. Plant Genetic Resources 16: 394–396.

Chochai A, Leitch IJ, Ingrouille MJ, Fay MF. 2012. Molecular phylogenetics of *Paphiopedilum* (Cypripedioideae; Orchidaceae) based on nuclear ribosomal ITS and plastid sequences. Botanical Journal of the Linnean Society 170: 176–196.

Cox AV, Pridgeon AM, Albert VA, Chase MW. 1997. Phylogenetics of the slipper orchids (Cypripedioideae: Orchidaceae); Nuclear rDNA sequences. Plant

Systematics and Evolution 208: 197–223. https://doi.org/10.1007/BF00985442

Cox AV, Abdelnour GJ, Bennett MD, Leitch IJ. 1998. Genome size and karyotype evolution in the slipper orchids (Cypripedioideae: Orchidaceae). American Journal of Botany 85: 681.

Cribb P. 1997. The Genus *Paphiopedilum*. Portland, Oregon: Timber Press.

Cribb P. 1998. The Genus *Paphiopedilum*. Kew: Royal Botanical Gardens/Borneo: Natural History Publications.

de Bruyn M, Stelbrink B, Morley RJ, Hall R, Carvalho GR, Cannon CH, van den Bergh G, Meijaard E, Metcalfe I, Boitani L, Maiorano L, Shoup R, von Rintelen T. 2014. Borneo and Indochina are major evolutionary hotspots for Southeast Asian biodiversity. Systematic Biology 63: 879–901. https://doi.org/10.1093/sysbio/syu047

Duncan RE, Macleod RA. 1949. The chromosomes of the continental species of *Paphiopedilum* with solid green leaves. American Orchid Society Bulletin 18: 84–89.

Duncan RE, Macleod RA. 1950. The chromosomes of *Eremantha tesselata*. American Orchid Society Bulletin 19: 137–142.

Ehrendorfer F. 1980. Polyploidy and distribution. In: Lewis WH (ed.) Polyploidy: Biological Relevance. New York: Plenum Press.

Govaerts R, Caromel A, Dhanda S, Davis F, Pavitt A, Sinovas P, Vaglica V. 2018. CITES Appendix I Orchid Checklist. Kew, Surrey: Royal Botanic Gardens and Cambridge: UNEP-WCMC.

Jones K. 1998. Robertsonian fusion and centric fission in karyotype evolution of higher plants. The Botanical Review 64: 273–289. https://doi.org/10.1007/BF02856567

Kamemoto H, Sagarik R, Dieutrakul S. 1963. Karyotypes of *Paphiopedilum* species of Thailand. Kasetsart Journal 3: 69–78.

Karasawa K. 1979. Karyomorphological studies in *Paphiopedilum* (Orchidaceae). Bulletin of the Hiroshima Botanical Garden 2: 1–149.

Karasawa K, Aoyama M. 1980. Karyomorphological studies on three species of *Paphiopedilum*. Bulletin of the Hiroshima Botanical Garden 3: 69–74.

Karasawa K, Aoyama M. 1988. Karyomorphological studies on two species of *Paphiopedilum*. Bulletin of the Hiroshima Botanical Garden 10: 1–6.

Karasawa K. 1982. The Genus *Paphiopedilum*. Hiroshima: Hiroshima Botanical Garden.

Karasawa K.1986. Karyomorphological studies on nine taxa of *Paphiopedilum*. Bulletin of the Hiroshima Botanical Garden 8: 23–42.

Karasawa K, Saito K. 1982. A revision of the genus *Paphiopedilum* (Orchidaceae). Bulletin of the Hiroshima Botanical Garden 5: 1–69.

Karasawa K, Tanaka R. 1980. C-banding study on centric fission in the chromosome of *Paphiopedilum*. Cytologia 45: 97–102.

Karasawa K, Tanaka R. 1981: A revision of chromosome numbers in some hybrids of *Paphiopedilum*. Bulletin of the Hiroshima Botanical Garden 4: 1–8.

Koopowitz H. 2000. A revised checklist of the genus *Paphiopedilum.* Orchid Digest 64: 155–179.

Lan T, Albert VA. 2011. Dynamic distribution patterns of ribosomal DNA and chromosomal evolution in *Paphiopedilum*, a lady's slipper orchid. BMC Plant Biology 11: 126. https://doi.org/10.1186/1471-2229-11-126

Lee Y-I, Chang F-C, Chung M-C. 2011. Chromosome paring affinities in interspecific hybrids reflect phylogenetic distances among lady's slipper orchids (*Paphiopedilum*). Annals of Botany 108: 113–121. https://doi.org/10.1093/aob.mcr114

Lee Y-I, Chung M-C, Sydara K, Souliya O, Aphay SL. 2017. Taxonomic placement of *Paphiopedilum rungsuriyanum* (Cypripedioideae; Orchidaceae) based on morphological, cytological and molecular analyses. Botanical Studies 58: 16. https://doi.org/10.1186/s40529-017-0170-1

Lee Y-I, Yap JW, Izan S, Leitch IJ, Fay MF, Lee F-C, Hidalgo O, Dodsworth S, Smulders MJM, Gravendee B, Leitch AR. 2018. Satellite DNA in *Paphiopedilum* subgenus *Parvisepalum* as revealed by high throughput sequencing and fluorescent in situ hybridization. BMC Genomics 19: 578. https://doi.org/10.1186/s12864-018-4956-7

Li DM, Wu W, Zhang D, Liu XR, Liu XF, L YJ. 2015. Floral transcriptome analyses of four *Paphiopedilum* orchids with distinct flowering behaviors and development of simple sequence repeat markers. Plant Molecular Biology Reporter 33: 1928–1952.

Lindley J. 1840. The genera and species of orchidaceous plants. London: Ridgways, Piccadilly.

Liu ZJ, Chen XQ, Cribb PJ. 2009. *Paphiopedilum* Pfitzer. In: Wu ZY, Raven P (eds.) Flora of China. Vol. 25. Beijing: Science Press; St. Louis: Missouri Botanical Garden Press.

Manton I. 1950. The spiral structure of chromosomes. Biological Reviews 25(4): 486-508. https://doi.org/10.1111/j.1469-185X.1950.tb00770.x

Mirzaghaderia G, Marzangi K. 2015. IdeoKar: an ideogram constructing and karyotype analyzing software. Caryologia 68(1): 31–35.

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J. 2000. Biodiversity hotspots for conser-

vation priorities. Nature 403: 853–858. https://doi.org/10.1038/35002501

Praptosuwiryo TNg, Darnaedi D. 2008. Cytological observation on fern genus *Pteris* in the Bogor Botanic Gardens. Buletin Kebun Raya Indonesia 11(2): 15-23.

Rankou H, Averyanov L. 2015. *Paphiopedilum armeniacum*. The IUCN Red List of Threatened Species 2015: e.T46695A3010661. https://doi.org/10.2305/IUCN.UK.20152.RLTS.T46695A3010661.en

Robertson RB. 1916. Chromosome studies. I. Taxonomic relationships shown in the chromosomes of tettigidae and acrididae: V-shaped chromosomes and their significance in acrididae, locustidae, and gryllidae: Chromosomes and variation. Journal of Morphology 27(2): 179–331. https://doi.org/10.1002/jmor.1050270202

Tsai C-C, Liao P-C, Ko Y-Z, Chen C-H, Chiang Y-C. 2020. Phylogeny and historical biogeography of *Paphiopedilum* Pfitzer (Orchidaceae) based on nuclear and plastid DNA. Frontiers in Plant Science 11: 126. https://doi.org/10.3389/fpls.2020.00126

Windham MD, Yatskievych G. 2003. Chromosome studies of cheilanthoid ferns (Pteridaceae: Cheilanthoideae) from the western United States and Mexico. American Journal of Botany 90(12): 1788–1800. https://doi.org/10.3732/ajb.90.12.1788

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Phytotoxicity, cytogenotoxicity and antimicrobial potential of extracts with gold-silver bimetallic nanoparticles obtained from pteridophyte spores

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Abstract. Investigating the toxicity of naturally occurring or synthesized nanoparticles for various applications is absolutely necessary for environmental protection and safety use. The aim of these research was to investigated the phytotoxicity, cytogenotoxicity and antibacterial potential of the extracts with gold-silver bimetallic nanoparticles (Au-Ag NPs) obtained from green synthesis in *Asplenium scolopendrium* L. and *Dryopteris filix-mas* (L.) Schott spores extracts. To our knowledge, this is the first report of the Au-Ag NPs phytosynthesis based on extracts obtained from fern spores. UV-Vis spectroscopy analysis of the samples revealed the maximum absorbance, characteristic of samples with bimetallic nanoparticles, which varied depending on the Au:Ag ratio. Energy-dispersive X-ray spectroscopy confirmed the presence and distribution of Au, Ag and other chemical elements. The presence of specific secondary metabolites in the extracts that helped in NPs biosynthesis stimulated growth processes. Good results

were recorded for some *Dryopteris filix-mas* samples, correlated with a significantly increased mitotic index. Cell viability decreased significantly in three of the nanoformulations. Only extracts with Au-Ag NPs showed antimicrobial effect against *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633 and *Escherichia coli* ATCC 8739. The testing of the antibacterial potential of these extracts must be extended to other bacterial strains and other microorganisms, the search of new antimicrobial resources being an urgent necessity nowadays.

Keywords: spore extracts, *Asplenium scolopendrium* L., *Dryopteris filix-mas* (L.) Schott, phytosynthesis, gold-silver nanoparticles, phytotoxicity, cell viability, cytogenotoxicity, antibacterial potential.

INTRODUCTION

Green synthesis is a promising substitute for traditional synthesis methods. Among different green synthesis methods, the use of unicellular and multicellular biological entities for obtaining nanoparticles (NPs) represents one of the most promising routes (Ettadili et al. 2022). Plants produce alkaloids, flavonoids, carbohydrates, polymers, proteins, and numerous antioxidants that are efficiently used in NPs synthesis (Patel et al. 2021), because they are involved in the bioreduction of metal salts (Nasrollahzadeh et al. 2019).

According to Chatterjee et al. (2019), among Cryptogames, algae and bryophytes are the most used to obtain nanoparticles, while ferns are little investigated. Various organs of ferns can be used to obtain the extracts needed for phytosynthesis, the spores being considered in recent years (Soare and Șuțan 2018).

The antioxidant potential and medicinal value of fern species determinate their selection for the synthesis of AgNPs and AuNPs (Makarov et al. 2013). By reducing $AgNO_3$ and $HAuCl_4$ in *Adiantum philippense* extracts, AuNPs and AgNPs were obtained (Sant et al. 2013) and Kunjiappan et al. (2015) reported the synthesis of AuNPs in *Azolla microphylla* extract.

The investigation of the phytotoxicity of NPs contributes to the establishment of their toxicity profile (USEPA 2005). Seed germination, root and stem growth, and seedling biomass are the morphophysiological parameters frequently used in such studies (Drăghiceanu et al. 2019; Pathipati et al. 2018). The biochemical parameters and those related to the cytogenotoxicity of NPs (Drăghiceanu et al. 2019) are often added to the morphophysiological ones, because if the NPs or the aggregates of NPs are small, they can penetrate the cell and interact with different cellular components and induce metabolic or genetic changes (Pathipati et al. 2018).

Silver has been considered an antimicrobial agent since ancient times. The antimicrobial activity of AgNP is influenced by two important factors: the high stability of dispersion and the release of Ag ions (Harada et al. 2018). AgNPs can effectively eliminate pathogenic bacteria, and by forming alloys with other noble metals (e.g Au) the stability of these materials can be significantly improved while maintaining antibacterial activity (Qin et al. 2021).

Au-Ag NPs showed increased antimicrobial, antioxidant and anticancer activities (Godipurge et al. 2016) compared to monometallic NPs due to the interactions between the two metals that occur in fine structures determining surfaces with new characteristics (Latif-ur-Rahman et al. 2015).

The first aim of this study was to determine the ability of aqueous extracts obtained from fern spores to produce bimetallic nanoparticles (Au-Ag NPs). Secondly, we sought to characterize the extracts with NPs by UV-Vis spectroscopy (UV-Vis), scanning transmission electron microscopy coupled with energy dispersive spectroscopy (STEM-EDX), X-Ray diffraction (XRD), and test to establish their phytotoxicity, cytogenotoxicity, and antibacterial potential.

MATERIALS AND METHODS

Obtaining extracts from fern spores

The spores used for the extracts were obtained from different mature individuals plants of *Asplenium scolopendrium* L. (A) and *Dryopteris filix-mas* (L.) Schott (D) from Vâlsan Valley (Argeș, Romania). The voucher specimens were recorded in the herbarium collection of the Argeș County Museum (*Asplenium scolopendrium* – 11.331, *Dryopteris filix-mas* – 11.330) (Soare et al. 2021). The ratio between plant material (spores) and solvent (distilled water) was 1:100 (g/mL). The micrometric dimensions of the spores which form a fine powder did not require grinding of the biological material. The spores and solvent were maintained in contact at room temperature (15 °C) for 5 days then filtered. For AuNPs and AgNPs phytosynthesis, we used plant extracts (DAM, AAM), 0.1 mM $HAuCl_4$ and 1 mM $AgNO_3$, the two reagents being added in 1:1 and 1:10 proportions (AA 1:1, AA 1:10, DA 1:1, DA 1:10) (Fierăscu et al. 2017b). The experimental variants are presented in Table 1.

Table 1. Experimental variants.

Variants	Content	Dilution
Control	Distilled water	-
DAM D10	Aqueous extract of D spores	10
DAM D100		100
DA1:1 D10	Aqueous extract of D spores with bimetallic nanoparticles (Au-Ag 1:1)	10
DA1:1 D100		100
DA1:10 D10	Aqueous extract of D spores with bimetallic nanoparticles (Au-Ag 1:10)	10
DA1:10 D100		100
AAM D10	Aqueous extract of A spores	10
AAM D100		100
AA1:1 D10	Aqueous extract of A spores with bimetallic nanoparticles (Au-Ag 1:1)	10
AA1:1 D100		100
AA1:10 D10	Aqueous extract of A spores with bimetallic nanoparticles (Au-Ag 1:10)	10
AA1:10 D100		100

Note: A - *Asplenium scolopendrium* L., D - *Dryopteris filix-mas* (L.) Schott.

Physicochemical characterization of extracts

Physicochemical characterization of extracts is performed by *UV-Vis*, *STEM-EDX* (HITACHI SU8230 microscope) and XRD (Rigaku SmartLab).

The formation of the Au-Ag NPs was examined using the PerkinElmer Lambda25 UV-Vis Spectrophotometer, in the range 370-600 nm for Au-Ag NPs (Fierăscu et al. 2017b) using a 10 mm quartz cuvette with optical path.

STEM-EDX was used to confirm the presence of Au and Ag, and to investigate particles shape and size distribution. For each extract a drop was poured on STEM sample holder (Ni grid with carbon support film) and dried for 24 hours in a desiccator. For each sample EDX area scans were performed in order to obtain chemical elemental information and confirm the Au and Ag presence. Also, EDX mapping have been obtained in order to investigate Au and Ag presence and distribution. STEM images provided information about particle's shape and size distribution (Soare and Șuțan 2018).

X-Ray diffraction. The solutions containing nanoparticles dispersions were prepared for analysis by deposition on the surface of the sample holder and evaporated at room temperature for 10-15 minutes before being subjected to analysis. The XRD analysis were performed using a 9 kW Rigaku SmartLab diffractometer (Rigaku Corp., Tokyo, Japan, 45 kV and 200 mA, CuKα radiation-1.54059 Å), in scanning mode $2\theta/\theta$, between 7° and 90° (2θ). The analyzes were performed using the Rigaku Data Analysis Software PDXL 2, database provided by ICDD.

Crystallite size was determined using the Debye-Scherrer equation:

$$D_P = \frac{K \times \lambda}{\beta \times cos\theta} \quad (1)$$

where *Dp* represents the average size of the crystallites, *K* - Scherrer constant (for cubic structures, $K = 0.94$), β - the width at half-height of the diffraction maximum, θ - Bragg angle, λ - wavelength (1.54059 Å in our case).

Assessment of the phytotoxic effect by Triticum test

The seeds of *Triticum aestivum* L., Miranda variety, were provided by Agricultural Research and Development Station Pitești, Albota, Romania. The seeds were hydrated in distilled water and immersed in the test solution for 1 hour. Then, the seeds were placed in Petri dishes on filter paper and periodically watered with distilled water. The Petri dishes were kept in the dark at 20±2 °C temperature and 20±3% humidity, until the measurements were made. Ten seeds were used for each variant.

After 4 days from the beginning of the experiment the root and stem length were measured and fresh and dry weight were determinate. The dry weight was established after keeping the plant material in the oven, at 80 °C, until a constant weight was obtained (Azooz et al. 2012).

The inhibition rate of the length of root and stem seedlings was calculated using formulas (2) provided by Ma et al. (2019):

$$\text{The inhibition rate} = [(Gm-Gx)/Gm]\times 100 \quad (2)$$

where Gm - values reached for the Control determined parameters, Gx - values reached for the determined parameters for the variants with extracts.

Evaluation of cytogenotoxic effects by Allium test

After removing the cataphylls and old roots, onion bulbs (*Allium cepa* L.) of about 3.5 cm diameter were placed with the discoidal stem in distilled water and kept in the dark for 48 h at room temperature. The bulbs with new roots were transferred to the test solution for 48 hours (Table 1). The roots were stored in 70% ethanol at 2-4 °C, after they were fixed 24 hours in Farmer's solution. The squash technique was used to display in a single layer the root tips cells hydrolysed with 1N HCl and stained with 2% aceto-orcein. To evaluate the cytogenetic activity of the extracts, approximately 3000 cells/experimental variant were analysed and the mitotic

index (MI), mitosis phase indices and the frequency of chromosomal aberrations were determined (Soare and Șuțan 2018).

Evaluation of cell viability by Evans Blue test

To establish the cell viability with Evans Blue staining, we used the protocols proposed by Chen et al. (2008), Vijayaraghavareddy et al. (2017) and Adamakis et al. (2019) with minor modifications. After the experimental treatment, 10 roots from each onion bulb were randomly selected. These were immersed for 15 minutes in 2 ml of 0.25% aqueous Evans Blue solution and then rinsed with distilled water to remove excess dye. The roots were kept in distilled water overnight at room temperature. To extract the dye, the apical parts of the roots (5 mm) were excised on the next day and placed in 2 ml of 1% aqueous solution of sodium dodecyl sulfate and kept in a water bath at 50 °C for one hour. For the quantitative determination of the absorbed dye, the absorbance at 600 nm was measured using T70+ UV-Vis Spectrophotometer.

Evaluation of antimicrobial activity

The antibacterial activity of the undiluted extracts with and without Au-Ag NPs was tested against standard bacterial strains (*Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633) (LTA, Italy) by Kirby-Bauer protocol on Mueller Hinton agar, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Radji et al. 2013). For the sterilized discs (6 mm diameter) 7 µl of extract was added and after the incubation period (24 h, 37 °C), the diameter of inhibition zones around the discs was determined. The measurements were performed considering the negative (distilled water, AD) and the positive (ampicillin, ATB) control. To determine minimal inhibitory concentration (MIC), binary serial dilutions of the tested extracts were performed (according to the CLSI standard, adapted broth dilution method), in which equal amounts of microbial inoculum were inoculated (Radji et al. 2013). Equal amounts of microbial inoculum (0,5 McFarland) and broth with decreasing amounts of extracts were incubated at 37 °C for 24 h. The results were appreciated by the unaided eye, considering the control (broth tube without extract, inoculated).

The interpretation of the results was made the next day considering the following aspects: clear culture medium – without bacterial development, hazy medium – bacterial development. The MIC value was determined by calculating the arithmetic mean of the last dilution with clear culture medium and the first dilution with hazy culture medium.

Statistical analysis

Data obtained after three repetitions of *Triticum* and *Allium* tests, Evans Blue and Kirby-Bauer protocols, were statistical analysed using IBM SPSS Statistics 23. The mean and standard error (SE) were calculated, and the averages were compared with Duncan's multiple comparison test.

RESULTS AND DISCUSSION

UV-Vis Spectroscopy

UV-Vis spectroscopy is a technique used to characterize nanoparticles of noble metals and is easy to apply (Hu and Xianyu 2021). UV-Vis analysis of the samples revealed that in the case of the extracts with bimetallic nanoparticles (Figure 1), the following peaks were obtained: 533 nm (AA1:1), 521 nm (DA1:1), and 441 nm (AA1:10, DA1:10). The peak value recorded for AA1:10 and DA1:10 is closer to monometallic Ag due to the higher proportion of Ag compared to Au. Our results are confirmed by other research. The formation of bimetallic NPs is highlighted by the appearance of a single band whose peak is located between that of the AuNPs and AgNPs (Tamuly et al. 2013; Garcia et al.

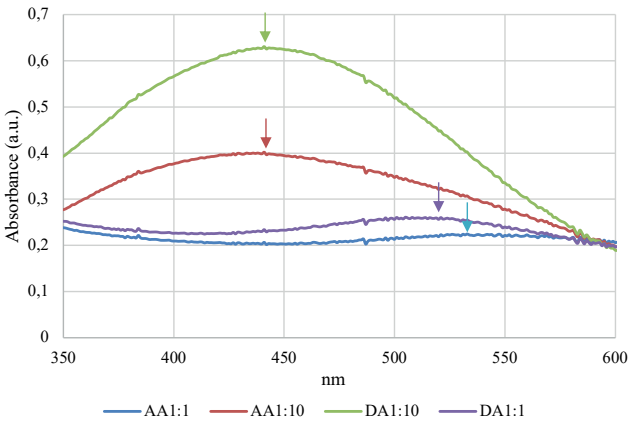

Figure 1. UV-Vis spectra of *Asplenium scolopendrium* L. and *Dryopteris filix-mas* (L.) Schott spores extracts with Au-Ag nanoparticles (AA1:1, AA1:10, DA1:1, DA1:10 sample). The arrow indicates the maximum absorbance obtained for the investigated samples.

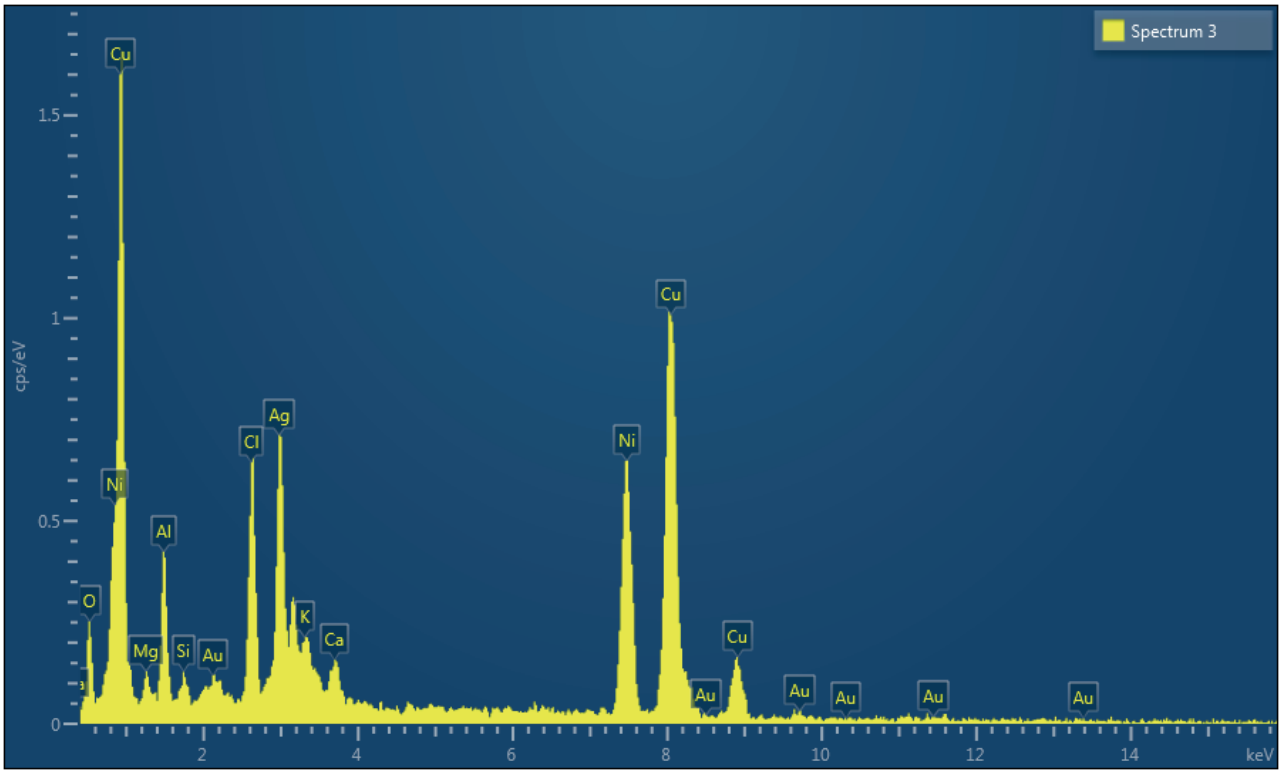

Figure 2. EDX spectra obtained for the *Asplenium scolopendrium* L. spores extract with Au-Ag nanoparticles (AA1:1 sample).

2014; Malathi et al. 2014). An example is for monometallic nanoparticles (AgNPs, AuNPs), as well as bimetallic ones (Ag-AuNPs) biosynthesized in extract obtained from the root of the medicinal plant *Plumbago zeylanica*, Salunke et al. (2014) obtained the maximum absorbance at 440 nm for Ag monometallics, at 570 nm for Au monometallics and at 540 nm for bimetallic ones. Çıplak et al. (2018) obtained a maximum absorbance at 410 nm for AgNPs and at 534 nm for AuNPs. In the case of bimetallic nanoparticles Ag67Au33, Ag50Au50 and Ag33Au67, the authors obtained different values of absorbance, depending on the Au:Ag ratio, respectively 412 nm, 519 nm and 523 nm.

In extracts with nanoparticles, the carbonyl group at 1635 cm^{-1} shows an increased intensity as a result of the capture/reduction of the metals (Drăghiceanu et al. 2021). It was also confirmed that the carbonyl group had stronger ability to bind with metal nanoparticles or act as stabilizing agents (Huleihel et al. 2002).

STEM-EDX Analysis

The investigation of the samples with EDX (Energy-dispersive X-ray spectroscopy) revealed the elemental composition (Su 2017), besides the elements added for obtaining the nanoparticles being highlighted and others (Cu, Ni, O, Al, Cl, Ca, Sn, K, and Mg), characteristic of the extracts, as it is observed from the Figures 2 and 3. The EDS mapping exposed the 2D presence and distribution of chemical elements within the investigated areas for all the samples, as can be seen for Au and Ag in *A. scolopendrium* (AA1:1) and *D. filix-mas* extracts (DA1:1) (Figure 4).

STEM analysis was used to investigate nanoparticles shape and size (Su 2017). The Au-Ag NPs obtained in the *A. scolopendrium* and *D. filix-mas* extracts had sizes between 5-39 nm for AA1:1 sample, 8-39 nm for AA1:10, 13-35 nm for DA1:1 and 15-35 nm for DA1:10 (Figure 5). Particle size between 4-94 nm and 2-78 nm, respectively, have been reported for AgNPs and AuNPs obtained by phytosynthesis assisted by various pteridophytes (Rao et al. 2021).

XRD Analysis

The crystallographic characteristics of the materials were evaluated from the diffraction pattern of the samples. Figure 6 presents the normalized spectra obtained for the four samples.

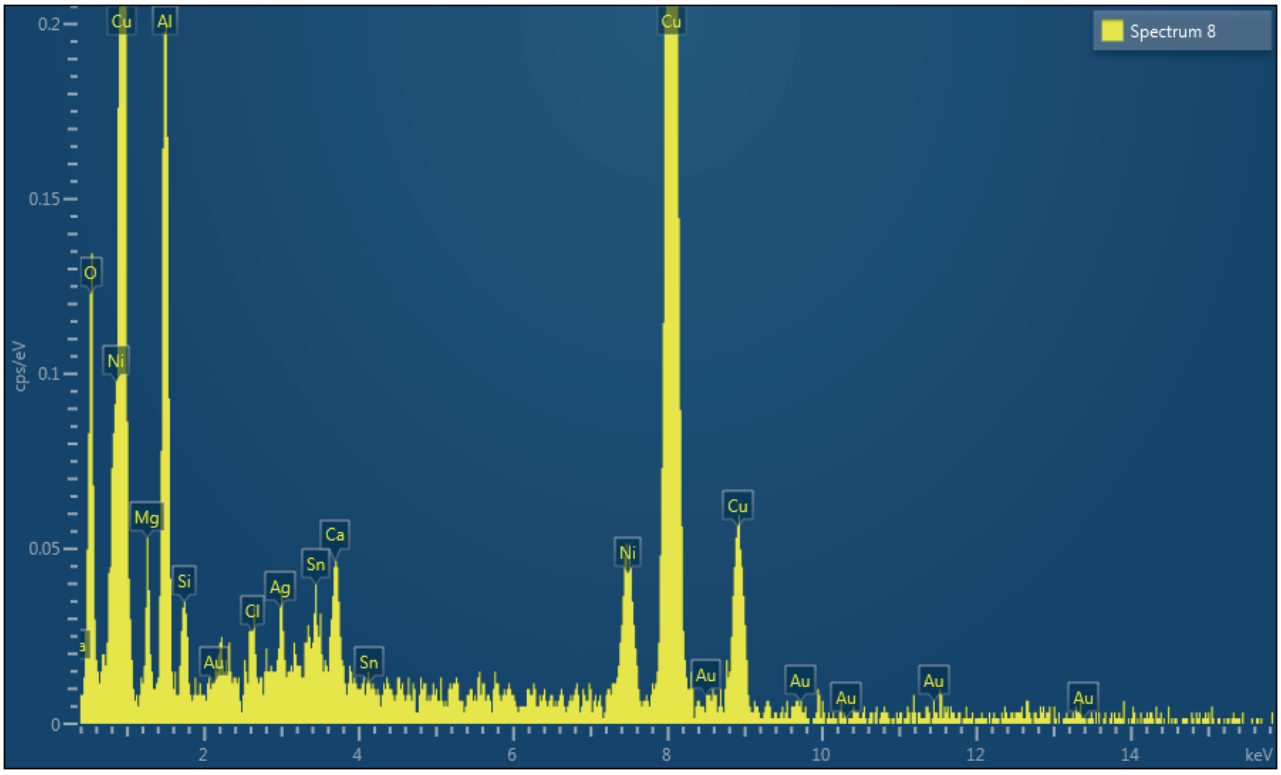

Figure 3. EDX spectra obtained for the *Dryopteris filix-mas* (L.) Schott spores extract with Au-Ag nanoparticles (DA1:1 sample).

The recorded spectra were interpreted using the dedicated software and the present phases were identified by comparison with corresponding ICDD entries. The results obtained are presented in Table 2. The identified phases were Au (ICDD card no. 00-004-0784), Ag (ICDD card no. 01-071-4613), Ag_2O (ICDD card no. 00-012-0793, marked with * on figure 6), and A_3O_4 (ICDD card no. 03-065-9750, marked with # on figure 6).

From XRD data it can be observed that all samples have a similar composition (although much well defined for sample DAA 1:10, while sample DAA 1:1 presents a much poorer defined spectra). Regarding the phases identified in the NPs solutions, it must be stated that the discrimination between Ag and Au is difficult, as the two metals exhibit similar diffraction patterns (the diffraction peaks overlapping). Also, the presence of different types of silver oxides was previously suspected to be due to oxidation of the NPs (Fierăscu et al. 2020; Șuțan et al. 2021).

Also, the identification must also consider the results obtained by other methods (especially UV-Vis and STEM-EDX). As such, samples with a lower Au:Ag ratio (AA 1:10 and DA 1:10) exhibit in the UV-Vis spectra a peak around 441 nm, which could be assigned to the presence of Ag_2O (Abouhaswa et al. 2022; Shume et al. 2020), although in our case the spectrum appears to have a small hypsocromic shift, which could be assigned to the presence of AgNPs (Bhui et al. 2009); other authors assign for the presence of Ag_2O a peak around 430 nm (Shume et al. 2020), which would imply a bathocromic shift of our spectra, which could be explained by a very small contribution of the AuNPs (suggested also by the shape of the UV-Vis spectra, slightly deformed towards higher wavelengths. The double silver oxide (Ag_3O_4) is most probably a secondary phase, formed either during reaction or during sample preparation for analysis, as other authors also noticed (Rajalakshmi et al. 2023) Samples AA and DA 1:1, presents specific UV-Vis peaks for AuNPs (above 520 nm). However, in the XRD spectra, these samples exhibit similar diffraction peaks as the other two samples. The presence of Au in the samples is also confirmed by the EDX mapping performed on the samples, as such, the most probably explanation for these two samples is that the silver / silver oxide phases are either masked by the AuNPs (in the form of core-shell structure, with the shell formed by AuNPs), which would allow the proheminent presence of AuNPs in the UV-Vis spectra, or by the oxidation of the AgNPs core during sample preparation. Our opinion, based on the analytic results, is that, for the samples

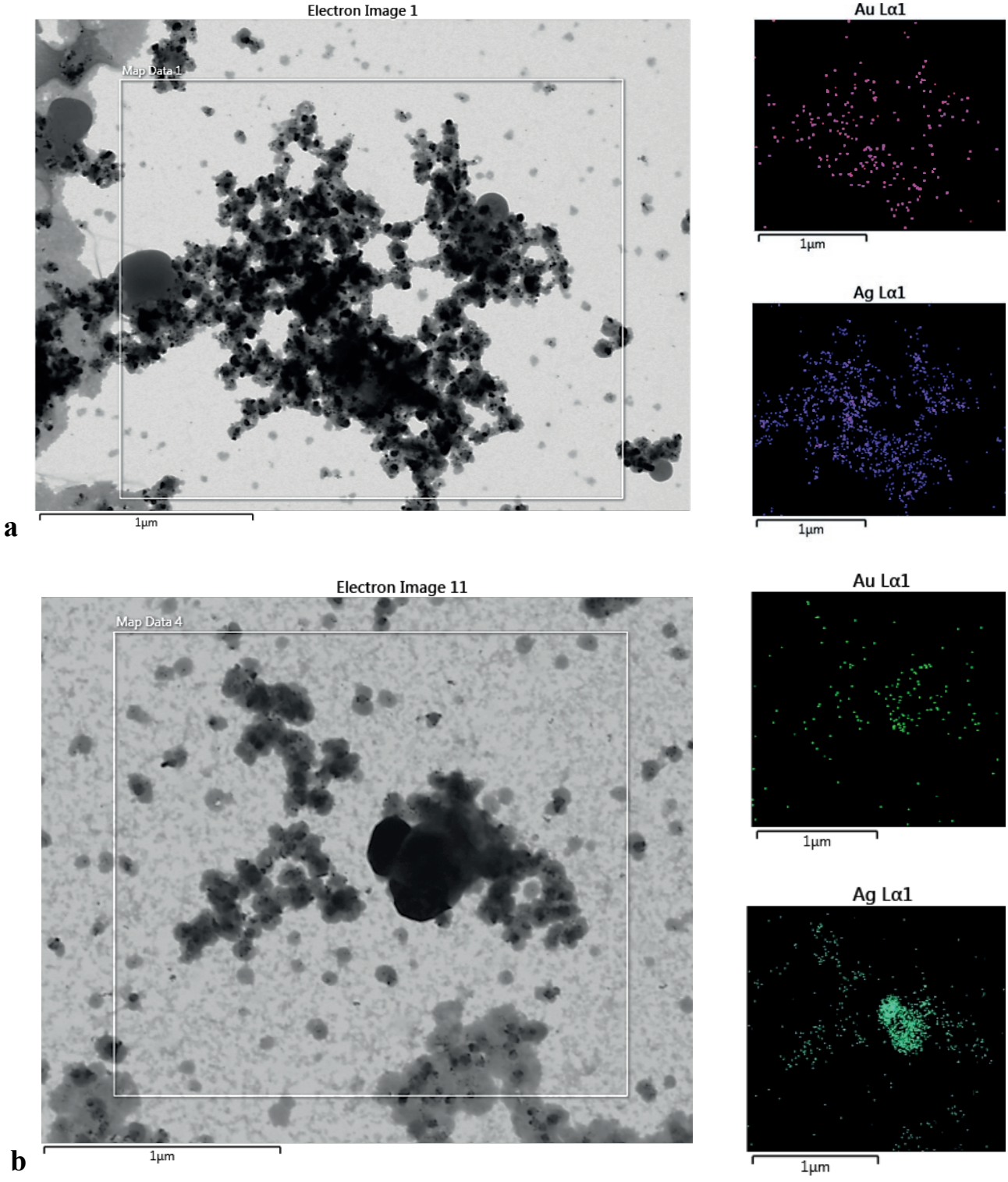

Figure 4. EDX-mapping - presence and distribution of chemical elements within the investigated areas (left): Au (right top) and Ag (right bottom) mapping in *Asplenium scolopendrium* L. spores extract with Au-Ag nanoparticles (AA1:1 sample) (**a**) and in *Dryopteris filix-mas* (L.) Schott (DA1:1 sample) (**b**).

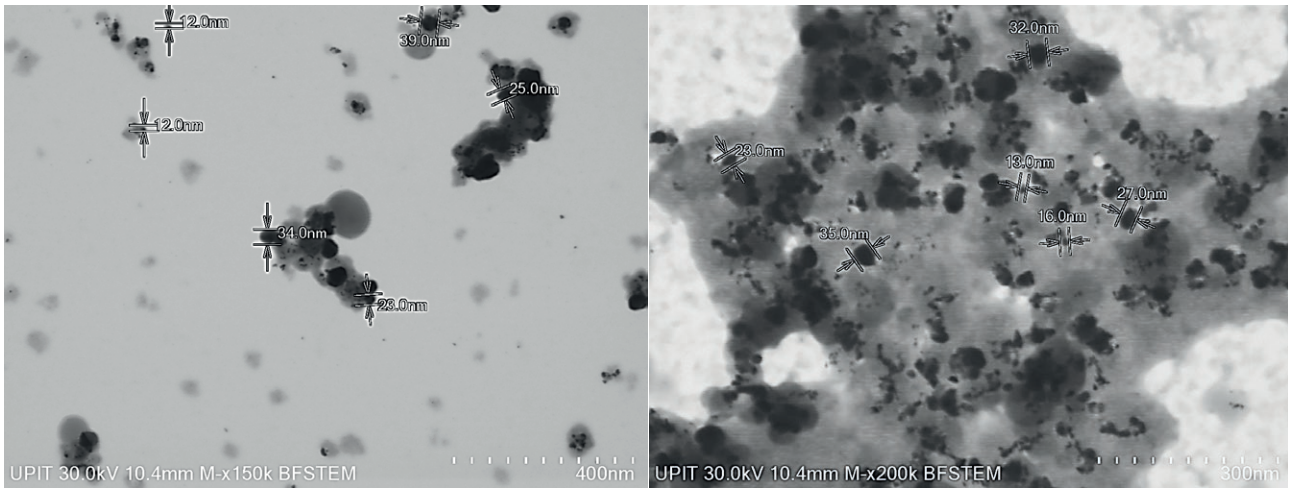

Figure 5. *Asplenium scolopendrium* L. extract with Au-Ag nanoparticles, AA1:1 sample. Au-AgNPs analysis in BFSTEM (x150k magnification) (left). *Dryopteris filix-mas* (L.) Schott extract with Au-Ag nanoparticles, DA1:1 sample. Au-AgNPs analysis in BFSTEM (x200k magnification) (right).

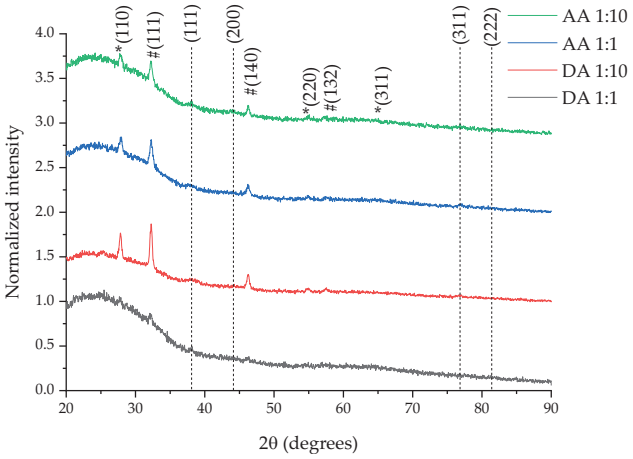

Figure 6. XRD spectra of the obtaining nanoparticles in *Asplenium scolopendrium* L. and *Dryopteris filix-mas* (L.) Schott spores extracts (blue AA 1:1, green AA 1:10, black DA1:1, red DA1:10).

with lower Au content (AA and DA 1:10) the silver/silver oxide nanoparticles with lower AuNPs content reprezent a majority phase, in the samples with a higher Au content (samples AA and DA 1:1), clusters of nanoparticles are formed, in which the AuNPs are found on the outer layer (thus contributing to the UV-Vis spectra), while the mixture of silver/silver oxide NPs found in the inner layer are re-arranged during sample preparation for XRD, which allows them to exhibit a much intense specific XRD peaks.

Assessment of the phytotoxic effects

The evaluation of phytotoxicity can be made by following some morphological, genetic, biochemical, physiological parameters, etc. *Triticum* test is frequently used for phytotoxicity studies in higher plants due to its advantages: quick results, simplified operative procedure, good reproducibility and repeatability and reduced costs (Drăghiceanu et al. 2019).

The extracts obtained, with and without NPs, stimulated the growth of the root and stem. Statistically significant differences were noticed for the root incubated in the DA1:10 D10, DA1:10 D100 samples and for the stem defined by DA1:10 D100 sample (Table 3). The insignificant growth inhibition observed in the DAM D100 sample, may be due to the decreased amount of bioactive substances, following the dilution of the sample. Fern spores contain many substances that also play a reserve role and in combating stress, such as lipids, proteins, and amino acids, such as proline, arginine, and some LEA-type proteins (late embryogenesis abundant), that promote embryo growth (López-Pozo et al. 2018). Except for the two abovementioned variants, the presence of NPs in extracts did not induce significant changes in root and stem growth. The presence of secondary metabolites in extracts cancels out the effect of nanoparticles (Zhang et al. 2021).

Wet and dry weight were not significantly influenced by the tested extracts. According to Jahn et al. (2010) the understanding of the extent of genetic variation for biomass traits in plants is limited. In a gen-

Table 2. XRD results obtained for the analyzed samples and the corresponding Miller indices.

Peak position (approx., 2q)	Sample/attribution				Crystallite size (determined using eq. (1)), nm			
	AA 1:1	AA 1:10	DA1:1	DA1:10	AA 1:1	AA 1:10	DA1:1	DA1:10
27.5	Ag_2O (110)	Ag_2O (110)	Ag_2O (110)	Ag_2O (110)	5.03	4.76	6.38	4.96
32.2	A_3O_4 (111)	A_3O_4 (111)	A_3O_4 (111)	A_3O_4 (111)	6.42	4.88	7.05	6.81
38	Au/Ag (111)	Au/Ag (111)	Au/Ag (111)	Au/Ag (111)	7.91	5.06	8.04	9.05
44.1	Au/Ag (200)	Au/Ag (111)	Au/Ag (111)	-	-	-	-	-
46.2	A_3O_4 (140)	A_3O_4 (140)	A_3O_4 (140)	A_3O_4 (140)	-	-	-	-
55	Ag_2O (220)	Ag_2O (220)	Ag_2O (220)	-	-	-	-	-
57.5	A_3O_4 (132)	A_3O_4 (132)	A_3O_4 (132)	A_3O_4 (132)	-	-	-	-
65.2	Ag_2O (311)	Ag_2O (311)	Ag_2O (311)	Ag_2O (311)	-	-	-	-
76.8	Au/Ag (311)	Au/Ag (311)	Au/Ag (311)	Au/Ag (311)	-	-	-	-
81.4	Au/Ag (222)	-	-	Au/Ag (222)	-	-	-	-

Table 3. The influence of aqueous spores extracts, with or without Au-Ag nanoparticles on *Triticum aestivum* L. parameters

Variants	Length (mm)		Phytotoxicity (%)		Weight (g)	
	Root	Stem	on root	on stem	Fresh	Dry
Control	35.93 ± 2.05^{bc}	13.93 ± 1.02^{cd}	0.00	0.00	0.82 ± 0.02^{a}	0.38 ± 0.00^{ab}
AAM D10	41.27 ± 1.76^{ab}	15.47 ± 0.81^{bcd}	-14.84	-11.00	0.87 ± 0.01^{a}	0.39 ± 0.01^{ab}
AAM D100	35.93 ± 1.65^{bc}	15.57 ± 0.69^{bcd}	0.00	-11.72	0.82 ± 0.02^{a}	0.36 ± 0.02^{ab}
DAM D10	39.40 ± 2.16^{abc}	16.77 ± 0.94^{bc}	-9.65	-20.33	0.77 ± 0.03^{a}	0.39 ± 0.02^{ab}
DAM D100	34.43 ± 2.03^{c}	13.80 ± 0.77^{d}	4.17	0.96	0.78 ± 0.04^{a}	0.35 ± 0.01^{b}
AA1:1 D10	38.20 ± 1.37^{abc}	14.90 ± 0.80^{bcd}	-6.31	-6.94	0.77 ± 0.03^{a}	0.39 ± 0.00^{ab}
AA1:1 D100	38.27 ± 1.54^{abc}	14.40 ± 0.62^{cd}	-6.49	-3.35	0.82 ± 0.06^{a}	0.38 ± 0.02^{ab}
DA1:1 D10	39.67 ± 1.92^{abc}	17.33 ± 0.59^{ab}	-10.39	-24.40	0.80 ± 0.02^{a}	0.40 ± 0.00^{a}
DA1:1 D100	38.77 ± 2.29^{abc}	14.13 ± 0.97^{cd}	-7.88	-1.44	0.84 ± 0.04^{a}	0.38 ± 0.02^{ab}
AA1:10 D10	39.70 ± 2.01^{abc}	14.50 ± 0.86^{bcd}	-10.48	-4.07	0.80 ± 0.05^{a}	0.37 ± 0.02^{ab}
AA1:10 D100	36.60 ± 1.90^{bc}	15.33 ± 0.92^{bcd}	-1.86	-10.05	0.78 ± 0.02^{a}	0.39 ± 0.02^{ab}
DA1:10 D10	44.17 ± 1.44^{a}	14.73 ± 0.76^{bcd}	-22.91	-5.74	0.75 ± 0.02^{a}	0.37 ± 0.00^{ab}
DA1:10 D100	43.63 ± 1.75^{a}	19.50 ± 1.43^{a}	-21.43	-39.95	0.80 ± 0.04^{a}	0.36 ± 0.01^{ab}

Data are shown as mean values ± SE of three replicates; a, b, c, d – interpretation of statistical significance and significant differences through Duncan's test, $p< 0.05$).

otype, wheat seed size and protein content are correlated with vigorous seedlings and higher yields biomass (Ries and Everson 1973). The seed we used in the experiment were from one wheat variety (Trivale), and the differences that appeared in dry biomass due to seed size and protein content are minimum. Hilty et al. (2021) consider that at the organ level and on short time scales, in our case wheat seeds and 4 days of growth, we can speak about growth in terms of one-dimensional elongation (roots, stems, leaves – for monocots) while growth as biomass accumulation should be used when we talk about plants and longer time scales. The biomass is the result of the photosynthesis minus photorespiration. Therefore, the biomass production can be enhanced by reducing photorespiratory losses (Peterhansel and Maurino 2010) or by increasing the photosynthesis rate and thus leaf area (Usuda 2004). Also, the leaf traits (thickness, size, shape, number etc.) are key factors in biomass production (Yang and Hwa 2008). In this study, in the short period of the experiment, the leaves didn`t appeared and the quantity of biomass produced by the stem was small. The small differences in biomass that were registered can be attributed to the depletion of the storage compounds Hilty et al. (2021), which was necessary for the root and stem growth.

Assessment of cytogenotoxic effects

Allium test is applied to determinate the effect of the plant extracts on the genetic material (Bonciu et al. 2018; Șuțan et al. 2016; Fierăscu et al. 2017a, b). The cytogenotoxic potential of various chemical agents can be assessed either by reducing or increasing of MI. In our study, statistical analysis revealed an insignificant increase in the frequency of mitotic cells in variants defined by aqueous extracts with or without Au-Ag NPs compared to the control. A significant increase of MI was determined by the aqueous extract of *D. filix-mas* spores DA1:10 D100 (Figure 7a). Similar results were reported by Șuțan et al. (2016) who found that ethanol extracts from *A. scolopendrium* leaves stimulated cell division in the root tips of *A. cepa*. In the root meristems *of A. cepa* exposed to actions of nanoparticles of iron oxide and copper, the MI increased by 10% and 5%, respectively, compared to the control, while AgNPs caused a decrease of 16% (Jasrotia et al. 2020). The stimulation of cell division and protein synthesis may be due to the electrostatic interaction of DNA and proteins caused by the penetration of AuNPs into the nucleoplasm (Balalakshmi et al. 2017). The increase in the MI in direct correlation with AuNP dose and without the appearance of chromosomal aberrations in onion meristematic root cells has also been reported by Gopinath et al. (2013). In this context, it is important to emphasize that the stimulation of cell division can have negative effects through an uncontrolled proliferation of cells (Hoshina et al. 2009).

After extract exposure of meristematic cells of *A. cepa*, prophases were observed with a higher frequency in variants with Au-Ag NPs 1:10 samples, regardless of the tested dilution. Significant differences in metaphase frequencies were observed between control and DA1:10 D100 (Figure 7b). The anaphase index does not exceed 23% in the root tip cells treated with aqueous extracts prior to or after Au-Ag NPs biosynthesis (Figure 7b) and the telophase had the lowest distribution in the observed population cells. Also, vagrants, micronucleus, binucleate cells and C-metaphase were identified in different samples. In the extracts with Au-Ag NPs DA1: 1 D10 and AA1: 10 D10, all five types of chromosomal aberrations mentioned were identified (Table 4; Figure 8). This increase in the frequency of aberrations compared to the control can be attributed to a high concentration of the phytosynthesized NPs.

Chromosomal aberrations observed by Palácio et al. (2021) in onion root meristem cells after exposure to AgNPs were delayed chromosomes, anaphase bridges, chromosome fragments and micronuclei. The authors appreciated that AgNPs disturbed the formation of the mitotic spindle, so that its partial or complete inactivation would cause the appearance of delayed chromosomes and the loss of genetic information. AgNPs can influence cell division by DNA degradation and depolymerization, their penetration into cells is facilitated by intracellular components (Kumari et al. 2009).

Rajeshwari et al. (2016) showed that AuNPs caused chromosome fragmentation, anaphase bridges, laggards, sticky chromosomes, and others abnormalities. However, it should be noted that the results found in the literature on the cytogenotoxic effect of nanoparticles depending on their concentration are contradictory. Thus, increasing the concentration of AgNPs induced a diminution in MI and the occurrence of various chromosomal aberrations, such as laggards, ring chromosomes, C-mitosis, chromosome fragmentation, nuclear membrane damage, multinuclear cells and chromatin bridges (Abdelsalam et al. 2019).

It has also been noticed that the MI and various nuclear abnormalities increased with the gradual reduction of the AgNPs diameter from 73 to 10 nm (Scherer et al. 2019). In our study, the higher frequency of chromosomal and nuclear aberrations recorded in the experimental AA1:1 D10 it may be due to the NPs with a diameter of 5-10 nm as the STEM-EDS analysis revealed.

Ahmed et al. (2018) stated that the MI modification and the induction of chromosomal aberrations could be due to the interference of the NPs with the DNA and/or the mitotic apparatus. However, we could not find similar results regarding the assessment of cytogenotoxicity of bimetallic nanoparticles on *Allium* assay.

Evaluation of cell viability by Evans blue test

The presence of Au-Ag NPs in extracts significantly influenced cell viability compared with experimental variants defined by the extracts without NPs. In the absence of NPs, the extract significant increase the cell viability compared to Control (Figure 9). At the variants DA 1:1 and AA 1:10 (diluted 10), the amount of Evans Blue absorbed by the roots of *Allium* was significantly higher than the amount obtained for the control. Zhang et al. (2019) considered that the absorption of a large amount of this dye is due to the damage of the cell membrane caused by the NPs. This situation is also confirmed by our results: for the variants with bimetallic nanoparticles dilution 100, the absorption of the Evans blue was smaller than that at dilution 10. The extracts without NPs (AAMD10, AAMD100, DAMD10, DAMD100) significantly increased the cell viability compared to Control due to the phytocompounds found in fern spores, com-

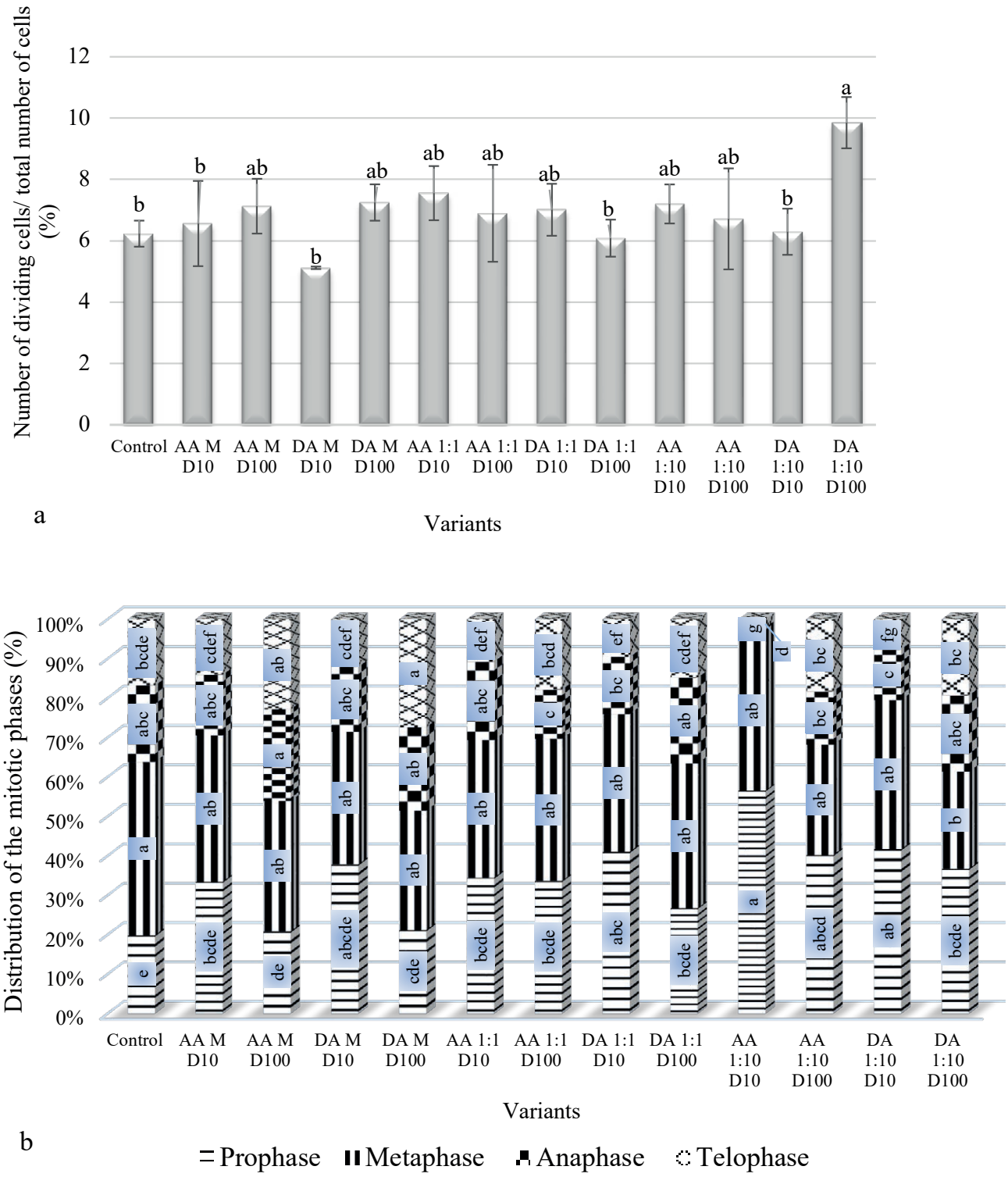

Figure 7. The influence of extracts on the mitotic index (a) and on the distribution on mitosis phase (b) in meristematic root cells of *Allium cepa* L. Data are shown as mean values ± SE of three replicates; a, b, c, d, e, f, g - interpretation of statistical significance and significant differences through Duncan's test, $p < 0.05$.

Table 4. Frequency of the main chromosomal aberrations in the meristematic root cells of *Allium cepa* L.

Variants	Chromosomal aberrations (%)				
	Anaphase bridges	Laggards	Micronucleus	Binucleate cells	C-metaphase
Control	-	1.33±1.33[a]	-	-	-
AAM D10	19.17±3.63[ab]	-	0.03±0.034[a]	-	-
AAM D100	11.57±6.43[ab]	-	-	-	-
DAM D10	11.01±2.44[ab]	-	-	0.07±0.07[a]	-
DAM D100	6.71±0.83[ab]	-	-	-	-
AA1:1 D10	22.78±13.62[a]	0.07±0.07[a]	-	0.07±0.07[a]	2.94±2.94[b]
AA1:1 D100	7.50±3.82 [ab]	0.85±0.85[a]	-	-	0.85±0.85[b]
DA1:1 D10	4.86±2.50 [ab]	2.98±1.50[a]	0.10±0.10[a]	0.21±0.06[a]	8.05±2.31[b]
DA1:1 D100	-	2.90±2.90[a]	-	-	4.35±2.51[b]
AA1:10 D10	1.75±3.03[b]	3.70±3.70[a]	0.10±0.06[a]	0.17±0.13[a]	65.74±5.63[a]
AA1:10 D100	-	-	-	-	7.69±7.69[b]
DA1:10 D10	1.45±2.51[b]	-	0.07±0.07[a]	0.03±0.03[a]	-
DA1:10 D100		-	-	0.17±0.07[a]	-

Data are shown as mean values ± SE of three replicates; a, b, c, d, e, f, g - interpretation of statistical significance and significant differences through Duncan's test, $p< 0.05$.

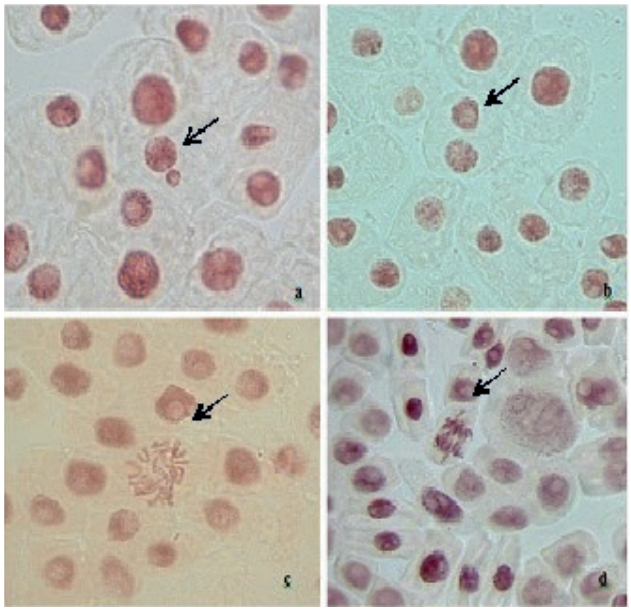

Figure 8. Chromosomal aberrations identified in the root meristem cells of *A. cepa* exposed to DA1:1 D10. (a) – micronucleus; (b) – binucleate cell; (c) – C-mitosis; (d) – vagrants.

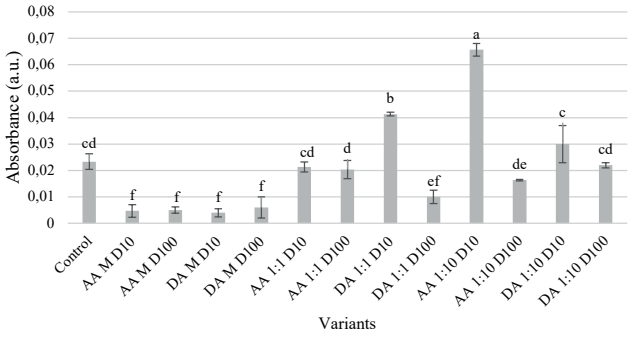

Figure 9. The influence of aqueous spores extracts, with or without Au-Ag nanoparticles, on cells viability. Data are shown as mean values ± SE of three replicates; a, b, c, d, e, f - interpretation of statistical significance and significant differences through Duncan's test, $p< 0.05$.

pounds that protect cells from stressors. The decrease of the amount of Evans blue absorbed by the roots after the treatment with various chemicals is explained by Baker and Mock (1994); they considered that the treatment can cause a large flow of electrolytes, but without necessarily causing cell death. Unlike us, after staining with Evans blue, Prajitha and Thoppil (2016) observed that aqueous extracts of *Amaranthus spinosus* L. induced cell death at the top of the *Allium* root, with the potential for membrane damage being significant. Regarding cell viability at the variants with nanoparticles diluted 100 times – we obtained similar (AA1:1, DA1:10) or greater values (DA1:1, AA1:10) compared to control. A similar situation was reported by Kannaujia et al. (2019) who studied the cell viability of the roots of two wheat varieties (HD-2967 and DBW-17) after exposure to AgNPs. After AgNPs exposure, the viability of wheat root cells assessed by Evans Blue staining was maximum in the case of wheat roots from the HD-2967 variety treated with AgNPs, while in the DBW-17 variety, the maximum viability of root cells was observed in the control and was close to that from the variant treated with AgNPs.

Antimicrobial activity of the extracts

The differences between the dimensions of inhibition zone induced by antibiotic and those induced by the aqueous extracts with bimetallic NPs are significant. The aqueous extract without NPs did not inhibit the development of bacterial strains (Figure 10b). Also, the samples DA1:10 and DA1:1 did not inhibit the growth of the *S. aureus* and *E. coli* strains. We consider that the characteristic bioactive substances of spores have a rather protective effect at the cellular level, even in the case of bacterial cells, the results being correlated with those obtained at the cell viability investigated by the Evans blue test. LEA protein, present in spores, provides protection against desiccation, osmotic, and oxidative stresses, the results being obtained using *E. coli* as an *in vivo* model to evaluate some LEA protein function (Saucedo et al. 2017).

Bimetallic nanoparticles inhibit the growth of *B. subtilis* ATCC 6633. The largest zone of inhibition of 7.17 mm was observed in the extracts with Au-Ag NPs 1:10, DA 1:10 sample (Figure 10a). A similar situation was observed for *E. coli* ATCC 8739, where the zone of inhibition was 8.83 mm in AA1:10 sample and 8 mm in DA1:10 sample (Figure 10a).

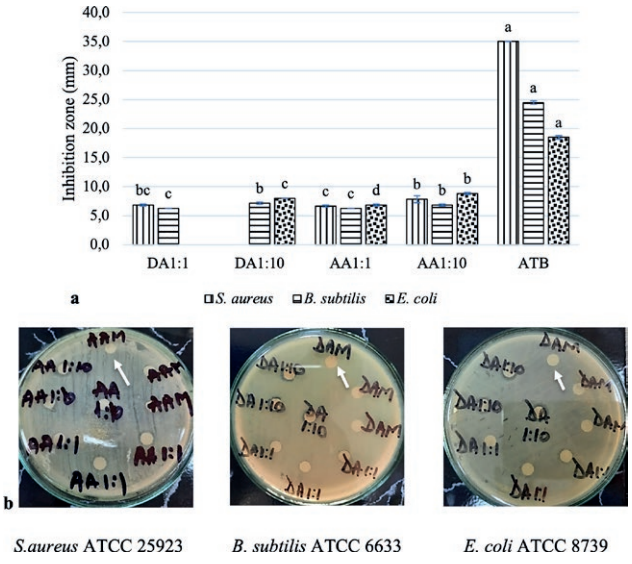

Figure 10. The antibacterial potential of the aqueous spores' extracts. Influence of extracts on *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 8739. The samples DA1:10 and DA1:1 did not inhibit the growth of the *S. aureus* and *E. coli* strain (**a**). Also, the aqueous extracts without nanoparticles had no antimicrobial effect, as seen (arrow) (**b**). The extract with nanoparticle may produce zone of inhibition like AA1:10 in *S. aureus*, DA1:10 in *B. subtilis*, and *E. coli* or may not produce inhibition zone in the tested strains, like DA1:1 in *E. coli*.

Table 5. Minimum inhibitory concentration (ml extract/ml medium).

Experimental variants	Bacterial strain		
	Bacillus subtilis A TCC 6633	*Escherichia coli* ATCC 8739	*Staphylococcus aureus* ATCC 25923
DA1:1	0.187	Nt	0.046
DA1:10	0.046	0.093	Nt
AA1:1	0.093	0.375	0.187
AA1:10	0.093	0.046	0.093

Note: Nt-not determined.

Aqueous extracts of spores of *A. scolopendrium* with Au-AgNPs had a higher antimicrobial efficiency in *S. aureus* ATCC 25923 than extracts of spores of *D. filix-mas* (Figure 10a). The influence of extracts on *S. aureus* ATCC 25923, *B. subtilis* ATCC 6633, *E. coli* ATCC 8739 are observed in Figure 10b.

MIC determined only for variants that had antimicrobial effect (Table 5) was between 0.046 ml extract for DA1:10 and AA1:10/ml medium in *B. subtilis* and *E. coli* and 0.187 ml extract for DA1:1 and AA1:1/ml medium in *B. subtilis* and *S. aureus* (Figure 11).

Au-Ag NPs strongly inhibited *B. subtilis* growth compared to control and monometallic nanoparticles; a similar situation was observed for *E. coli* (Reddy et al. 2012). The increase in the number of Ag ions released from bimetallic nanoparticles indicates that Au ions influence the oxidation of Ag atoms (Harada et al. 2018). Green-synthesized Ag-Au NPs exhibited promising antibacterial activity against *E. coli*, *B. subtilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *S. aureus* in a dose-dependent manner (Amina et al. 2020). The antibacterial potential of plant extracts with bimetallic Ag-Au NPs depends on particle size, shape, area and surface polarity, morphology, and plant specific compounds (Amina et al. 2020). Spore-specific bioactive compounds allowed the production of green bimetallic nanoparticles, which have superior properties to those obtained by chemical synthesis, being less phytotoxic, biocompatible, environmentally friendly, which might be due to the capping of biomolecule onto the surface of NPs (Panicker et al. 2020).

CONCLUSIONS

The aqueous extracts obtained from the spores of the native ferns *A. scolopendrium* and *D. filix-mas* constituted optimal media for the biosynthesis of Au-Ag

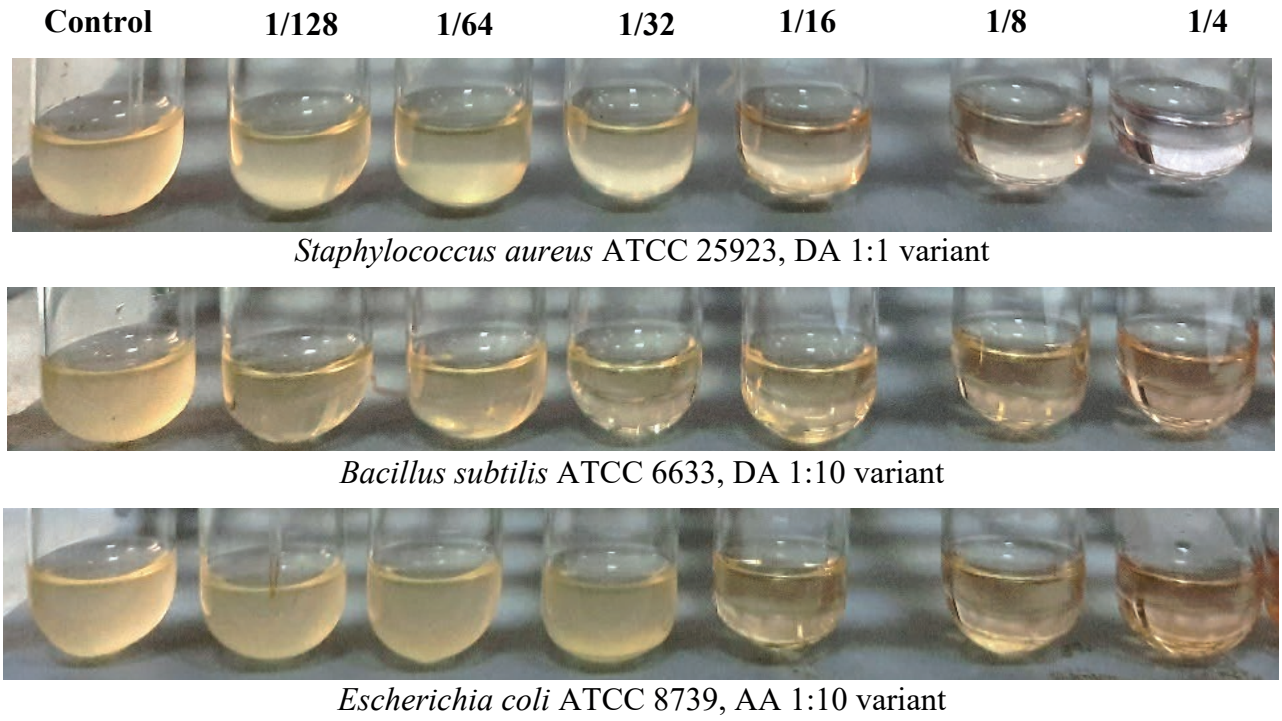

Figure 11. Aspects of MIC evaluation. Control (broth tube without extract), bacterial cultures obtained in various extract dilutions (1/128, 1/64, 1/32, 1/4).

NPs. The growth processes evaluated in the seedlings of *Triticum aestivum* were, in general, stimulated by both categories of extracts, with and without NPs, significant differences being obtained for those of *D. filix-mas*. The effect of stimulating the growth of axial organs was also confirmed by the results obtained in the *Allium* test. Extracts without NPs significantly improved cell viability, assessed by the Evans blue test, alongside the variant with NPs, DA1:1 D100. An antimicrobial effect was observed just for sample with bimetallic NPs, against all three bacterial strains: *S. aureus* ATCC 25923, *B. subtilis* ATCC 6633 and *E. coli* ATCC 8739. The sample with aqueous extract without NPs did not inhibit the development of bacterial strains. The increase in antibiotic resistance of microorganisms requires the discovery of new products with such properties, so it is useful to continue the research of less evaluated resources, such as fern spores.

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AUTHOR CONTRIBUTIONS

Conceptualization: OAL, LCS, NAŞ, IF, RCF, DN; experimental design and laboratory work: OAL, LCS,

IF, RCF, CMD, AP, CMP, CMT, LEV, ID, DN, DȘV, GC, FA, SOH, NAȘ; funding acquisition: OAL, NAȘ, IF, RCF. Writing, review and editing: all authors.

REFERENCES

Abdelsalam NR, Fouda MMG, Abdel-Megeed A, Ajarem J, Allam AA, El-Naggar ME. 2019. Assessment of silver nanoparticles decorated starch and commercial zinc nanoparticles with respect to their genotoxicity on onion. Int J Biol Macromol. 133:1008-1018. https://doi.org/10.1016/j.ijbiomac.2019.04.134

Abouhaswa AS, Almurayshid M, Almasoud F. Sayyed MI, Mahmoud KA. 2022. Examinations the optical, mechanical, and shielding properties of Ag_2O doped B_2O_3–Bi_2O_3–SrF_2–Na_2O glasses for gamma ray shield applications. Sci Rep. 12:3548. https://doi.org/10.1038/s41598-022-07450-7

Adamakis I-D, Eleftheriou E. 2019. Structural evidence of programmed cell death induction by tungsten in root tip cells of *Pisum sativum*. Plants 8(3):62. https://doi.org/10.3390/plants8030062

Ahmed B, Shahid M, Khan MS, Musarrat J. 2018. Chromosomal aberrations, cell suppression and oxidative stress generation induced by metal oxide nanoparticles (MONPs) in onion (*Allium cepa*) bulb. Metallomics 10(9):1315-1327. https://doi.org/10.1039/c8mt00093j

Amina M, Al Musayeib NM, Alarfaj NA, El-Tohamy MF, Al-Hamoud GA. 2020. Antibacterial and immunomodulatory potentials of biosynthesized Ag, Au, Ag-Au bimetallic alloy nanoparticles using the *Asparagus racemosus* root extract. Nanomat. 10(12): 2453. https://doi.org/10.3390/nano10122453

Azooz MM, Abou-Elhamd MF, Al-Fredan MA. 2012. Biphasic effect of copper on growth, proline, lipid peroxidation and antioxidant enzyme activities of wheat (*Triticum aestivum* cv. *Hasaawi*) at early growing stage. Austal J Crop Sci. 6(4):688-694.

Baker CJ, Mock NM. 1994. An improved method for monitoring cell death in cell suspension and leaf disc assays using Evans blue. Plant Cell Tiss Organ Cult. 39:7-12.

Balalakshmi C, Gopinath K, Govindarajan M, Lokesh R, Arumugam A, Alharbi NS, Kadaikunnan S, Khaled JM, Benelli G. 2017. Green synthesis of gold nanoparticles using a cheap *Sphaeranthus indicus* extract: Impact on plant cells and the aquatic crustacean *Artemia nauplii*. J Photochem Photobiol B: Biol. 173:598-605. https://doi.org/10.1016/j.jphotobiol.2017.06.040

Bhui DP, Bar H, Sarkar P, Sahoo GP, De SP, Misra A, 2009. Synthesis and UV-Vis spectroscopic study of silver nanoparticles in aqueous SDS solution. J Mol Liquids 145(1):33-37. https://doi.org/10.1016/j.molliq.2008.11.014

Bonciu E, Firbas P, Fontanetti CS, Wusheng J, Karaismailoğlu MC, Liu D, Menicucci F, Pesnya DS, Popescu A, Romanovsky AV, Schiff S, Ślusarczyk J, de Souza CP, Srivastava A, Sutan A, Papini A. 2018. An evaluation for the standardization of the *Allium cepa* test as cytotoxicity and genotoxicity assay. Caryologia 71(3):191-209. https://doi.org/10.1080/00087114.2018.1503496

Chatterjee A, Khatua S, Acharya K, Sarkar J. 2019. A green approach for the synthesis of antimicrobial bio-surfactant silver nanoparticles by using a fern. Dig J Nanomater Biostruct. 14(2):479-490.

Chen PY, Lee KT, Chi WC, Hirt H, Chang CC, Huang H.J. 2008. Possible involvement of MAP kinase pathways in acquired metal-tolerance induced by heat in plants. Planta 228(3):499-509. https://doi.org/10.1007/s00425-008-0753-x

Çıplak Z, Gökalp C, Getiren B, Yıldız A, Yıldız N, 2018. Catalytic performance of Ag, Au and Ag-Au nanoparticles synthesized by lichen extract. Green Process Synth 7:433-440. https://doi.org/10.1515/gps-2017-0074

Drăghiceanu OA, Fierăscu I, Fierăscu RC, Bradzis R, Dobrescu CM, Soare LC. 2019. Considerations regarding the *Triticum* phytotoxicity test. Curr Trends Nat Sci. 8(15):48-55.

Drăghiceanu OA, Șuțan AN, Dobrescu CM, Bătut-Andrei ND, Soare LC, Topală CM. 2021. Characterization in terms of composition and phytotoxicity of aqueous spores extract. Curr Trends Nat Sci. 10(20):53-60. https://doi.org/10.47068/ctns.2021.v10i20.008

Ettadili FE, Aghris S, Laghrib F, Farahi A, Saqrane S, Bakasse M, Lahrich S, El Mhammedi MA. 2022. Recent advanced in the nanoparticles synthesis using plant extract: applications and future recommendations. J Mol Struct. 1248:131538. https://doi.org/10.1016/j.molstruc.2021.131538

Fierăscu I, Milen IG, Ortan A, Fierăscu RC, Avramescu SM, Ionescu D, Șuțan A, Brînzan A, Ditu LM. 2017a. Phyto-mediated metallic nanoarchitectures *via Melissa officinalis* L.: synthesis, characterization and biological properties. Sci Rep. 7:12428. https://doi.org/10.1038/s41598-017-12804-7

Fierăscu RC, Milen IG, Fierăscu I, Ungureanu C, Avramescu SM, Ortan A, Georgescu MI, Șuțan, NA, Zanfirescu A, Dinu-Pirvu CE, Velescu BS, Anuta V. 2017b. Mitodepressive, antioxidant, antifungal and

anti-inflammatory effects of wild-growing Romanian native *Arctium lappa* L. (*Asteraceae*) and *Veronica persica* Poiret (*Plantaginaceae*). Food Chem Tox. 111:44-52. https://doi.org/10.1016/j.fct.2017.11.008

Fierăscu RC, Fierăscu I, Lungulescu EM, Nicula N, Somoghi R, Dițu LM, Ungureanu C, Șuțan AN, Drăghiceanu OA, Păunescu A, Soare LC. 2020. Phytosynthesis and radiation-assisted methods for obtaining metal nanoparticles. J Mat Sci. 55:1915-1932. https://doi.org/10.1007/s10853-019-03713-3

Garcia AG, Lopes PP, Gomes JF, Pires C, Ferreira EB, Lucena RGM, Gasparotto LHS, Tremiliosi-Filho G. 2014. Eco-friendly synthesis of bimetallic AuAg nanoparticles. New J. Chem. 38(7):2865-2873. https://doi.org/10.1039/c4nj00041b

Godipurge SS, Yallappa S, Biradar NJ, Biradar JS, Dhananjaya BL, Hedge G, Jagadish K, Hedge G. 2016. A facile and green strategy for the synthesis of Au, Ag, and Au-Ag alloy nanoparticles using aerial part of *R. hypocrateriformis* extract and their biological evaluation, Enz. Microb. Technol. 95:174-184. https://doi.org/10.1016/j.enzmictec.2016.08.006

Gopinath K, Venkatesh KS, Ilangovan R, Sankaranarayanan K, Arumugam A. 2013. Green synthesis of gold nanoparticles from leaf extract of *Terminalia arjuna*, for the enhanced mitotic cell division and pollen germination activity. Ind Crops Prod. 50:737-742. https://doi.org/10.1016/j.indcrop.2013.08.060

Harada A, Ichimaru H, Kawagoe T, Tsushida M, Niidome Y, Tsutsuki H, Sawa T, Niidome T. 2018. Gold-Treated Silver Nanoparticles Have Enhanced Antimicrobial Activity. Bull Chem Soc Japan. 92:297-301. https://doi.org/10.1246/bcsj.20180232

Hilty J, Muller B, Pantin F, Leuzinger S. 2021. Plant growth: the What, the How, and the Why. New Phytol. 232(1):25-41. https://doi.org/10.1111/nph.17610

Hoshina M, Marin-Morales M.A. 2009. Micronucleus and chromosome aberrations induced in onion (*Allium cepa*) by a petroleum refinery effluent and by river water that receives this effluent. Ecotoxicol Environ Saf. 72:2090-209. https://doi.org/10.1016/j.ecoenv.2009.07.002

Hu J, Xianyu Y. 2021. When nano meets plants: A review on the interplay between nanoparticles and plants. Nano Today 38:101143. https://doi.org/10.1016/j.nantod.2021.101143

Huleihel M, Salman A, Erukhimovich V, Ramesh J, Hammody Z, Mordechai S. 2002. Novel optical method for study of viral carcinogenesis *in vitro*. J Biochem Biophys Meth. 50:111-121. https://doi.org/10.1016/s0165-022x(01)00177-4

Jahn CE, Mckay JK, Mauleon R, Stephens J, McNally KL, Bush DR, Leung H, Leach JE. 2010. Genetic Variation in Biomass Traits among 20 Diverse Rice Varieties. Plant Physiol. 155(1):157-168. https://doi.org/10.1104/pp.110.165654

Jasrotia T, Chaudhary S, Kaushik A, Kumar R, Chaudhary GR. 2020. Green chemistry-assisted synthesis of biocompatible Ag, Cu, and Fe_2O_3 nanoparticles. Mat Today Chem. 15:100214. https://doi.org/10.1016/j.mtchem.2019.100214.2

Kannaujia R, Srivastava CM, Prasad V, Singh BN, Pandey V. 2019. *Phyllanthus emblica* fruit extract stabilized biogenic silver nanoparticles as a growth promoter of wheat varieties by reducing ROS toxicity. Plant Physiol Biochem. 142:460-471. https://doi.org/10.1016/j.plaphy.2019.08.008

Kumari M, Mukherjee A, Chandrasekaran N. 2009. Genotoxicity of silver nanoparticles in *Allium cepa*. Sci Total Environ. 407:5243-5246. https://doi.org/10.1016/j.scitotenv.2009.06.024

Kunjiappan S, Bhattacharjee C, Chowdhury R. 2015. Hepatoprotective and antioxidant effects of *Azolla microphylla* based gold nanoparticles against acetaminophen induced toxicity in a fresh water common carp fish (*Cyprinus carpio* L.). Nanomed J. 2(2):88-110. https://doi.org/10.7508/nmj.2015.02.002

Latif-ur-Rahman, Shah A, Khan SB, Asiri AM, Hussain H, Han C, Qureshi R, Ashiq MN, Zia MA, Ishaq M, Kraatz HB. 2015. Synthesis, characterization, and application of Au-Ag alloy nanoparticles for the sensing of an environmental toxin, pyrene. J Appl Electrochem. 45(5):463-472. https://doi.org/10.1007/s10800-015-0807-2

López-Pozo M, Fernández-Marín B, García-Plazaola JY, Ballesteros D. 2018. Desiccation Tolerance in Ferns: From the Unicellular Spore to the Multi-tissular Sporophyte. In Fernández H. editor. Current Advances in Fern Research. Springer, Cham; p. 401-426, https://doi.org/10.1007/978-3-319-75103-0_19

Makarov VV, Love AJ, Sinitsyna OV, Makarova SS, Yaminsky IV, Taliansky ME, Kalinina NO. 2014. "Green" nanotechnologies: synthesis of metal nanoparticles using plants. Acta Nat. 6(1):35-44.

Malathi S, Ezhilarasu T, Abiraman T, Balasubramanian S. 2014. One pot green synthesis of Ag, Au and Au-Ag alloy nanoparticles using isonicotinic acid hydrazide and starch. Carb Pol. 111:734-743. https://doi.org/10.1016/j.carbpol.2014.04.105

Nasrollahzadeh M, Sajjadi M, Sajadi SM, Issaabadi Z. 2019. Green Nanotechnology. In An Introduction to Green Nanotechnology, Nasrollahzadeh, M, Sajadi, S.M., Sajjadi, M., Issaabadi, Z., Atarod, M., Editors, Elsevier, Interface Science and Technology, pp 145-198. https://doi.org/10.1016/b978-0-12-813586-0.00005-5

Palácio SM, de Almeida JCB, de Campos ÉA, Veit MT, Ferreira LK, Deon MTM. 2021. Silver nanoparticles effect on *Artemia salina* and *Allium cepa* organisms: influence of test dilution solutions on toxicity and particles aggregation. Ecotoxicol. 30(5):836-850. https://doi.org/10.1007/s10646-021-02393-7

Panicker S, Ahmady IM, Han C, Chehimi M, Mohamed AA. 2020. On demand release of ionic silver from gold-silver alloy nanoparticles: fundamental antibacterial mechanisms study. Mat Today Chem. 16:100237. https://doi.org/10.1016/j.mtchem.2019.100237

Patel AK, Gupta D, Singh A, Mishra VK, Sharma NK. 2021. Green - synthesized nanoparticles for treatment of wastewater: an environmentally sustainable pollution remediation technology. In Sustainable Environmental Clean-up, Green Remedation; Mishra VK, Kumar A, Eds., Elsevier, pp. 29-70. https://doi.org/10.1016/B978-0-12-823828-8.00002-5.

Pathipati UR, Kanuparthi PL. 2018. Biological and Phytotoxic Impacts of a Nanomaterial. In *Phytotoxicity of Nanoparticles*; Faisal, M., Saquib Q., Alatar, A.A., Al-Khedhairy, A.A., Eds., Springer Cham., pp. 229-240. https://doi.org/10.1007/978-3-319-76708-6_9

Peterhansel C, Maurino VG. 2010. Photorespiration Redesigned. Plant Physiol, 155(1):49-55. https://doi.org/10.1104/pp.110.165019

Prajitha V, Thoppil JE. 2016. Cytotoxic and apoptotic activities of extract of *Amaranthus spinosus* L. in *Allium cepa* and human erythrocytes. Cytotechnol. 69(1):123-133. https://doi.org/10.1007/s10616-016-0044-5

Qin Z, Zheng Y, Wang Y, Du T, Li C, Wang X, Jiang H. 2021. Versatile roles of silver in Ag-based nanoalloys for antibacterial applications, Coord Chem Rev. 449, 214218. https://doi.org/10.1016/j.ccr.2021.214218

Radji M, Agustama RA, Elya B, Tjampakasari CR. 2013. Antimicrobial activity of green tea extract against isolates of methicillin-resistant *Staphylococcus aureus* and multi-drug resistant *Pseudomonas aeruginosa*. Asian Pac J Trop Biomed. 3(8):663-667. https://doi.org/10.1016/S2221-1691(13)60133-1

Rajalakshmi TU, Sheeba H, Doss A, Veerabahu R, Sivagnanam A, Alfarraj S, Alharbi SA, Subbiah J, Mariselvam R. 2023. Synthesis of silver nanoparticles from natural derived embelin compound and their uses in mercury degradation under solar light. Mat Res Express. 10(5):055502. https://doi.org/10.1088/2053-1591/acd2ad

Rajeshwari A, Roy B, Chandrasekaran N, Mukherjee A. 2016. Cytogenetic evaluation of gold nanorods using *Allium cepa* test. Plant Physiol Biochem. 109:209-219. https://doi.org/10.1016/j.plaphy.2016.10.003

Rao KJ, Korumilli T, Jakkala S, Singh K, Vidya K. 2021. Optimization of the one-step green synthesis of silver and gold nanoparticles using aqueous *Athyrium filix femina* extract using the Taguchi method. BioNanoSci. 11:915-922. https://doi.org/10.1007/s12668-021-00909-3

Reddy RP, Varaprasad K, Narayana Reddy, N., Mohana Raju, K., Reddy, N.S. 2012. Fabrication of Au and Ag bi-metallic nanocomposite for antimicrobial applications. J Appl Polymer Sci. 125(2):1357-1362. https://doi.org/10.1002/app.35192

Ma C, He M, Zhong Q, Ouyang W, Lin C, Liu X. 2019. Uptake, translocation and phytotoxicity of antimonite in wheat (*Triticum aestivum*), The Sci Total Environ. 669:421-430. https://doi.org/10.1016/j.scitotenv.2019.03.145

Ries SK, Everson EH. 1973. Protein content and seed size relationships with seedling vigor of wheat cultivars. Agron J. 65:884-886.

Salunke GR, Ghosh S, Kumar RJS, Khade S, Vashisth P, Kale T, Chopade S, Pruthi V, Kundu G, Bellare JR, Chopade BA, 2014. Rapid efficient synthesis and characterization of silver, gold, and bimetallic nanoparticles from the medicinal plant *Plumbago zeylanica* and their application in biofilm control. Int J Nanomed. 9:2635-2653. https://doi.org/10.2147/ijn.s59834

Sant DG, Gujarathi TR, Harne SR, Ghosh S, Kitture R, Kale S, Chopade BA, Pardesi KR. 2013. *Adiantum philippense* L. frond assisted rapid green synthesis of gold and silver nanoparticles. J Nanopart. 182320. https://doi.org/10.1155/2013/182320

Saucedo AL, Hernández-Domínguez EE, de Luna-Valdez LA, Guevara-García AA, Escobedo-Moratilla A, Bojorquéz-Velázquez E, del Río-Portilla F, Fernández-Velasco DA, Barba de la Rosa AP. 2017. Insights on Structure and Function of a Late Embryogenesis Abundant Protein from Amaranthus cruentus: An Intrinsically Disordered Protein Involved in Protection against Desiccation, Oxidant Conditions, and Osmotic Stress. Front Plant Sci. 8:497. https://doi.org/10.3389/fpls.2017.00497

Scherer MD, Sposito JCV, Falco WF, Grisolia AB, Andrade LHC, Lima SM, Machado G, Nascimento VA, Gonçalves DA, Wender H, Oliveira SL, Caires ARL. 2019. Cytotoxic and genotoxic effects of silver nanoparticles on meristematic cells of *Allium cepa* roots: A close analysis of particle size dependence. Sci Total Environ. 660:459-467. https://doi.org/10.1016/j.scitotenv.2018.12.444

Shume WM, Murthy HCA, Zereffa EA. 2020. A Review on Synthesis and Characterization of Ag_2O Nano-

particles for Photocatalytic Applications J Chem. 5039479. https://doi.org/10.1155/2020/5039479

Soare LC, Şuţan AN. 2018. Current trends in pteridophyte extracts: from plant to nanoparticles. In *Current Advances in Fern Research*, Fernandez H., Eds., Springer, pp. 329-357. https://doi.org/10.1007/978-3-319-75103-0_16

Soare LC, Păunescu A, Dobrescu CM, Neblea MA, Dorobăţ LM. 2021. Ferns – a valuable plant resource in modern research. In *Development of plant extracts and innovative phytosynthesized nanostructures mixtures with phytotherapeutic applications, in order to reduce biocenotic stress in horticultural crops,* Fierăscu RC, Fierăscu I, Soare LC, Eds., Ruse Press, pp. 27-44.

Su D. 2017. Advanced electron microscopy characterization of nanomaterials for catalysis. *Green Energy Environ.* 2(2):70-83. https://doi.org/10.1016/j.gee.2017.02.001

Şuţan NA, Fierăscu I, Fierăscu RC, Manolescu DS, Soare LC. 2016. Comparative analytical characterization and *in vitro* cytogenotoxic activity evaluation of *Asplenium scolopendrium* L. leaves and rhizome extracts prior to and after Ag nanoparticles phytosynthesis. Ind Crops Prod. 83:379-386. https://doi.org/10.1016/j.indcrop.2016.01.011

Şuţan NA, Fierăscu I, Şuţan C, Soare LC, Neblea AM, Somoghi R, Fierăscu RC. 2021. In vitro mitodepressive activity of phytofabricated silver oxide nanoparticles (Ag_2O-NPs) by leaves extract of *Helleborus odorus* Waldst. & Kit. ex Willd, Mat Lett. 286:129194. https://doi.org/10.1016/j.matlet.2020.129194

Tamuly C, Hazarika M, Borah SC, Das MR, Boruah MP. 2013. *In situ* biosynthesis of Ag, Au and bimetallic nanoparticles using *Piper pedicellatum* C.DC: Green chemistry approach. Coll Surf B: Biointerf. 102:627-634. https://doi.org/10.1016/j.colsurfb.2012.09.007

USEPA, 2005. Nanotechnology white paper external review draft. https://www.epa.gov/osa/pdfs/EPA_nanotechnology_white_paper_external_review_draft_12-02-2005

Usuda H. 2004. Evaluation of the effect of photosynthesis on biomass production with simultaneous analysis of growth and continuous monitoring of CO_2 exchange in the whole plants of radish, cv Kosena under ambient and elevated CO_2. Plant Prod Sci. 7(4):386-396. https://doi.org/10.1626/pps.7.386

Vijayaraghavareddy P, Adhinarayanreddy V, Ramu SV, Sreeman S, Udayakumar M. 2017. Quantification of membrane damage/cell death using Evan's blue staining technique. Bioprot. 7(16): e2519. https://doi.org/10.21769/BioProtoc.2519

Yang XC, Hwa CM. 2008. Genetic modification of plant architecture and variety improvement in rice. Heredity 101:396-404

Zhang H, Chena S, Jia X, Huang Y, Ji R, Zhao L. 2021. Comparation of the phytotoxicity between chemically and green synthesized silver nanoparticles. Sci Total Environ. 752:142264. https://doi.org/10.1016/j.scitotenv.2020.142264

Zhang WY, Wang Q, Li M, Dang F, Zhou DM. 2019. Nonselective uptake of silver and gold nanoparticles by wheat. Nanotoxicol. 13(8):1073-1086. https://doi.org/10.1080/17435390.2019.1640909

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Antibacterial power of Pomegranate extracts against Beta-Lactamase producing *Escherichia coli*

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Abstract. Herbs, as the pomegranate (*Punica granatum* L.)(*P. granatum*), has significant chemical constituents with distinct pharmacological properties. These chemicals confer neuroprotective, antioxidant, anticancer, anti-inflammatory, and antibacterial properties to the plant. Pomegranate has specific components that enable its pharmacological actions; one of the functions of pomegranate extracts is to deactivate what is called extended spectrum beta-lactamase (ESBL) that makes *Escherichia coli* (*E. coli*) resistant to standard antibiotics. Twenty *E. coli* strains identified as beta-lactamase producers, the strains isolated from urine samples collected from patients with symptomatic urinary tract infection (UTI) and molecularly characterised using 16S rDNA. The study evaluated the antibiotic sensitivity and antibacterial activities of acetone and ethanolic pomegranate leaf and peel extracts, assessing their antimicrobial susceptibility against nineteen antibiotics. The ehanolic peel (EP) and leave (EL) extracts showed inhibitory potential inhibition zones spanning (9.0-12.6-18.3 mm) against *E. coli* pathogen producing extended-spectrum beta-lactamase as compared with (10.2-15.3 mm) inhibition scale exhibited by acetone peel (AP) and leave (AL) extracts treatment. Pomegranate leaves and peel extracts contain bioactive compounds with antioxidant, antimicrobial, and other biological effects, and can be fractionated for the identification of new antibacterial bioactive compounds for the development of drugs against ESBL- *E. coli*, in addition to their synergy with antibiotics for combination therapy that may have effective management and treatment of multidrug-resistant infections such as urinary tract infection.

Keywords: *Punica granatum*, *E. coli*, beta-lactamase enzyme, antibacterial activities, antimicrobial assay, DNA sequencing.

1. INTRODUCTION

In addition to use plants as food, shelter, and building materials, plants were traditionally employed as medicinal herbs. Herbs were defined as those with medicinal properties (Kunle *et al*., 2012). The World Health Organization (WHO) defines herbal medicines as plants whose parts are used to treat and prevent illnesses in humans and animals (Msomi and Simelane 2019). They include active agents; either alone or in combination can be used in the treatment and management of chronic conditions, such as cancer and cardiovascular difficulties. Researchers have resorted to herbal drugs as alternatives to standard therapy methods as no bad side effects (Sutan *et al*., 2023). These unique natural constituents, herbal medication significantly contributed to disease management (Sutan *et al*., 2023; Kam and Neergheen 2022; Facciola and Stephen 1990). The usage of herbal medicines has grown in popularity and awareness around the world because to their inexpensive cost, little to non-existent side effects, and lack of bacterial resistance. In addition, these herbs are easily accessible as they are readily available. The therapeutic properties of *P. granatum* shrub are attributed to the extract from its numerous sections, which has been shown to be beneficial in treating and preventing the pathogenic *E. coli* strain (Stover and Mercure 2007). Pomegranate is a deciduous plant that grows between five and eight metres in height. It is a member of the Lythraceae family. Pomegranate was traditionally regarded as a symbol of life, fertility, health, wisdom, immortality, and longevity (Loizzo *et al*., 2019). Pomegranate is planted primarily in South Asia and the Middle East. Today, it is widely cultivated in dry and semiarid places due to its adaptability to harsh climates (Stover and Mercure 2007). Each anatomical component of the plant has a unique pharmacological and toxicological function. This includes the peel, seed, flower, juice, leaf, bark, and root. It is an edible fruit with a thick, reddish skin, a rounded hexagonal shape, and a 5 to 12 cm diameter. The fruit contains approximately 600 seeds, each wrapped in a water-filled aril (pulp) of varying hues ranging from white to deep purple to deep crimson. The fruit's edible portion, the seeds, can be utilised in baking, cooking, and beverage preparation (Loizzo *et al*., 2019). However, the Pomegranate has been widely utilised by numerous nations and civilizations for the treatment of numerous ailments (Kim and Choi 2009). Previous research indicates that the chemical constituents contained in Pomegranate seeds, bark, flowers, pericarp, and roots confer the plant with a variety of therapeutic effects. In addition, it has antioxidant, anti-atherosclerotic, anti-inflammatory, neuroprotective, anticancer, antimicrobial, antihypertensive, anti-teratogenic, stimulant, antidiabetic, antiviral, antifungal, anthelmintic, nephron-protective, wound-healing, and antiulcer properties.

In addition, its chemical ingredients help combat respiratory disorders and erectile dysfunction (Kim and Choi 2009). While Pomegranate seeds contain estrogenic chemicals, estradiol and oestrone, and the fruit has been widely used to treat microbial infections, acidosis, diarrhoea, haemorrhage, helminthic infection, respiratory ailments, and dysentery, they do not have estrogenic properties themselves (Ricci *et al*., 2006). Juice and dried pericarp of the fruit have been used to treat colic, headache, colitis menorrhagia, piles, oxyuriasis, diuretics, allergic dermatitis, acne, and oral illnesses (Berthe *et al*., 2013). *Escherichia coli* (*E. coli*) is a bacteria found in the intestines of animals (Denamur *et al*., 2021). The majority of *E. coli* strains are digestively helpful and harmless, some are dangerous and pathogenic (Khalid and Andreoli 2019). Pathogenic strains of *E. coli* bacteria are spread mostly by contaminated human-to-human contact, contaminated food or water consumption, and contaminated surfaces (Denamur *et al*., 2021; Al-Sarraj *et al*., 2021a; Alotibi *et al*., 2022). Infections of the urinary tract, gastrointestinal tract, and respiratory system are caused mainly by pathogenic *E. coli* strains. *E. coli* infections are characterised by moderate diarrhoea, abdominal discomfort, bloody diarrhoea, and renal failure (Qi *et al*., 2022). In order to treat or prevent *E. coli* infections, one must adhere to food safety and sanitary standards and practise proper hygiene.

Beta-lactams are the most often used antibiotics for the treatment of urinary tract infections (Flores-Mireles *et al*., 2015); nevertheless, the rise of multiple-drug resistant (MDR) bacteria, particularly beta-lactamase (ESBL) generating pathogens, is a huge concern to the global healthcare system (Shaikh *et al*., 2015; Hashem *et al*., 2017; Heidari *et al*., 2017; Houri *et al*., 2017; Motamedifar *et al*., 2015). The prevalence of *E. coli* bacteria that produce beta-lactamase enzymes has increased recently on a global scale (Pourakbari *et al*., 2012; Kazemian *et al*., 2016). It inhibits the effectiveness of certain beta-lactam antibiotics, such as penicillin and cephalosporins, by degrading them. By generating beta-lactamase enzymes, bacteria acquire antibiotic resistance. Enzymes degrade the structure of beta-lactam antibiotics, rendering their antibacterial effects inactive (Qi *et al*., 2022; Amanulla and Sundaram 2019). Therefore, treating bacterial infections caused by *E. coli* generating beta-lactamase becomes problematic. The proliferation of antibiotic-resistant bacteria has become a global health concern, compelling researchers to investigate

alternate preventative and therapeutic measures (Huang *et al*., 2019; Chauhan *et al*., 2020). The strategies include the creation of novel antibiotics, the use of natural chemicals such as plant extracts, and the improvement of sanitation practises to avoid the spread of infectious diseases (Amanulla and Sundaram 2019; Hashim and Pharma 2013). Leaves of *P. granatum* contain chemical substances with the ability to suppress the actions of *E. coli* strains producing beta-lactamase. With the correct concentration of the extract, pathogenic *E. coli* will eventually be incapable of degrading the structure of the antibiotics. Consequently, the molecules of the antibiotic continue to combat bacterial infections. This study comprehensively evaluates the antibacterial impacts of *P. granatum* leaves and peel extracts on *E. coli* extended spectrum β-Lactamase isolate and the antibiotic susceptibility patterns of *E. coli* isolate. Additionally, it provides a comparison between the concentrations of *P. granatum* extractions and seeks to find the most effective concentration and its synergy with antibiotics that may have potential for future treatment of multi-drug resistant infections.

2. MATERIALS AND METHOD

All of the chemicals and antibiotics employed were of analytical quality, purchased from Sigma-Aldrich, and were utilised without further purification.

2.1. Herbal material and microorganisms

The pomegranate plant used in this investigation was collected in Taif, Saudi Arabia. The Hematology department of the Faculty of Medicine at King Abdulaziz University in Jeddah, Saudi Arabia, reported urine samples from twenty *E. coli* isolates isolated from urine samples of twenty-one patients with urinary tract infections (UTIs) and one healthy control individual in June 2021. The bacteria were cultured in Luria-Bertani (LB) medium with 200 rpm shaking at 37 °C for twenty-four hours before being stored at 4 °C. Next, we cultivated bacterial isolates on LB agar overnight at 37 °C for 24 hours. After incubation, the cultures were inspected to determine if considerable growth had occurred. All collected strains were sequenced for the 16S rDNA gene to validate the culture-based identification of bacteria. The optical density of bacterial cultures was set to 600 nm (OD600) with sterile 0.1 M saline solution for evaluating Disk Diffusion, Antibiotic Susceptibility Assay, and the antibacterial activity of pomegranate leaves and peel extracts.

2.2. Antibiotics

Amikacin AK 30mcg, Ampicillin AMP 10mcg, Cefrtriaxone CTR 30 mcg, Amoxyclav AMC (Amoxycillin+Clavulinic Acid) (Augmentin) 30mcg, Ceftazidime CAZ 30 mcg, Cefoperazone CPZ 75 mcg, Netilmicin NET 30 mcg, Cefuroxime CX.

2.3. Antibiotics sensitivity assay for bacteria

Twenty bacterial isolates were tested against eight distinct drugs to determine their susceptibility. The bacteria were cultured overnight in nutritional broth for activation. They were then determined by measuring optical density (OD) at 600 nm with a UV-visible spectrophotometer and subsequently standardised to 0.1 McFarland standards (3107 CFU/mL). Later, the bacterial suspension was dispersed on nutrient agar plates. The agar was then covered with antibiotic discs and incubated at 37 °C for 18-20 hrs. Later, the sizes of the zones of inhibition were measured and reported.

2.4. Preparing pomegranate extracts

The peel and leaves of the pomegranate were gathered by hand, separated, chopped into little pieces, then rinsed with tap water and then distilled water to eliminate any suspended soil and other particles. The samples were then air-dried in the shade at room temperature to preserve their freshness. An electric mill was used to grind the dried samples into a fine pomegranate powder. The powder was kept in airtight containers at 4 °C until usage. 50 g of the powder was soaked in 500 ml of ethanol and 500 ml of acetone for 5 minutes, and then the mixture was swirled continuously at 200 rpm for 72 hrs to dissolve. After that, filtration was performed in three stages. Initially, the fluid was filtered through numerous layers of gauze to remove the suspended plant fibres and particles. Afterward, it was filtered with Whitman No. 1 paper. Using a rotary evaporator, the filtrate was evaporated. The storage solution was made by dissolving 500 mg/ml of the extracted powder in 40 ml of 2 percent diluted Dimethyl Sulfoxide (DMSO).

2.5. Antibacterial activity of pomegranate extracts

Using the agar well diffusion method, the antibacterial activity of pomegranate leaf extract was determined. 20-25 ml of autoclaved Muller–Hinton agar media was poured into a sterile petri dish, which was then allowed to harden. Spread the bacterial suspensions on the plate

using a sterile cotton swab. Each plate includes five holes that were drilled with a 6mm cork borer and filled with 100µl of extracts of varying concentrations. As a negative control, 30 percent diluted DMSO without pomegranate leaf extract was used. The plate was left for 10 minutes to allow the chemicals to pre-diffuse into the agar. The plates were then incubated at 37 °C for 24 hours. The diameter of the inhibitory zone was then measured in millimetres.

2.6. Beta-Lactamase production assay

In order to determine the beta-lactamase synthesis of all examined *E. coli* isolates, broth samples were point-injected onto Mueller-Hinton agar (MHA) containing 1 percent starch and incubated at 37 degrees Celsius. After 24 hours of incubation, the plates were refilled with PBS containing potassium iodide, iodine, and penicillin. The creation of distinct colorless zones around the *E. coli* growth indicates the production of -lactamase enzymes, which convert Penicillin to penicilloic acid and iodine to iodide, as shown by the decolorization of the starch iodine complexes.

2.7. Molecular characterization of bacteria

DNA extraction and amplification of 16S rRNA genes

Total genomic DNA was extracted from 20 urine samples collected from patients with symptomatic urinary tract infection using a slightly modified version of the Azcárat-Peril and Raya (2001) protocol, in which 1 ml of pure bacteria culture grown overnight in NB was transferred to a 1.5 ml tube, and the tube was centrifuged at 10000 rpm, 4°C for 5 minutes. After discarding the supernatant, 200 µl of TES buffer is added and vortexed thoroughly. Then, 20µl of lysozyme (10 mg/ml) was added and thoroughly mixed using a vortex. Two hours were spent incubating the mixture at 37°C in a water bath. Each sample received 20µl of proteinase K (10 mg/ml), which was then vortexed and incubated at 37°C for two hours. The liquid was chilled for five minutes, then 250 µl of sodium acetate was added. After 5 minutes of centrifugation at 8000 rpm and 4°C, the top zone was carefully transferred to a new, clean Eppendorf tube. 250 µl of chloroform: isoamyl (24:1) was added to the mixture, which was then mixed by hand and centrifuged at 8000 rpm, 4°C for five minutes. The aqueous phase was then transferred to a fresh Eppendorf. Equal volume of isopropanol was added to the mixture, which was then stored overnight at -20 degrees Celsius. The following day, the solution was centrifuged at 10000 rpm for 5 minutes, after which the liquid zone was discarded and the pellet was allowed to dry at ambient temperature for 10 minutes before being suspended in 50 µl of distilled water. DNA from each bacterial isolate was served as template for amplification of the 16S rRNA genes using the following universal primers: 27F (5'- AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3').

For the PCR, a total volume of 50µl was utilized, along with 2µlof 10 pmol of each appropriate primer and 25µl of Master Mix (GoTaq® Green Master Mix, 2X, Promega). Add 2l of DNA and use DEPEC-treated water to adjust the volume of the final PCR mixture to 50 µl. The 16S rRNA gene was amplified using a thermal cycler (applied biosystems TM Veriti TM 96-Well Thermal Cycler) under the following conditions: initial denaturation at 95 °C for 4 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing for 45 seconds, and extension at 72 °C for 60 seconds, and a final extension at 72 °C for 10 minutes.

2.8. Sequence analysis and phylogeny of E. coli strains

The consensus 16S rDNA amplicon sequences of *E. coli* isolates were modified and subjected to a BLAST search using the NCBI database http://blast.ncbi.nlm.nih.gov/Blast.cgi to assign presumptive identity with similar sequences. On the basis of sequence similarity measurements and inferences of phylogenetic trees, each bacterial isolate was assigned to its operational taxonomic unit (OTU). Where necessary, alignments were edited by hand. The pathogenic *E. coli* nucleotide sequences were submitted to GenBank for assignment of accession numbers. The acquired sequences were separated into distinct datasets in order to examine the evolutionary relationships between species and families. The NJ technique was employed to create phylogenetic trees, whereas MEGA software, version 10.0 (Tamura *et al.*, 2011) was utilised to undertake molecular evolutionary studies.

2.9. Statistical analysis

This study's statistical analysis was conducted using the SPSS software (Version 26.0). All analyses were conducted in triplicate using a one-way ANOVA analysis of variance with a significance level of p 0.05, and the results were represented as the mean standard deviation.

3. RESULTS

3.1. Molecular identification of the E. coli strains

All 20 bacterial isolates were identified molecularly by amplification of the 16S rRNA gene using universal primers. According to the PCR results, the 16S rRNA gene sequences of the selected isolates were amplified successfully from extracted template DNA. Using gel electrophoresis, the PCR products of the 16S rRNA genes of these isolates yielded bands with around 1500 bp (Figure 1).

DNA sequencing was used to identify the 20 examined pathogenic *E. coli* strains at the molecular level by analysing the 16S rRNA gene. The GenBank was accessed to obtain the accession numbers for all of the bacterium isolates listed in Table 1, and MEGA software was used to optimise the sequences that were obtained. Using the NCBI public database, nucleotide similarities between 16S rRNA gene sequences and those of GenBank-recognized species were analysed. The results indicated that the isolated microorganisms were *E. coli*. The sequences of the acquired *E. coli* strains were 99.2–100% comparable to those of their closely related isolates previously deposited in GenBank. The homologous sequences from the BLAST search were utilised to construct a phylogenetic tree, demonstrating their relationship to *E. coli* strains, as demonstrated in the Figure 2.

This Original Tree's evolutionary history was inferred using the UPGMA method (Sneath and Sokal 1973), and the 500-replicate bootstrap consensus tree (Felsenstein 1985) is considered to represent the evolutionary history of the species examined (Felsenstein 1985). The collapsed branches correspond to partitions that are replicated in less than half of bootstrap replicates. Next to each branch is the percentage of 500 replicate trees in which the relevant taxa clustered together in the bootstrap test (Felsenstein 1985). Using the Maximum Composite Likelihood approach (Tamura *et al*., 2004) and the amount of base substitutions per site, the evolutionary distances were estimated. This analysis used 40 nucleotide sequences, and for each sequence pair, all ambiguous locations were eliminated (pairwise deletion option). The final dataset contained 1030 locations in total. In MEGA X, evolutionary analyses were undertaken (Kumar *et al*. 2018).

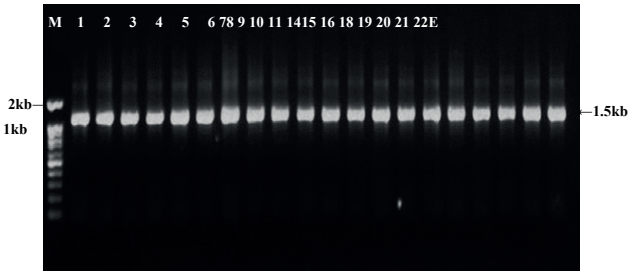

Figure 1. Agarose gel electrophoresis of PCR products for 16s rDNA gene. Lane M: 2kb DNA size marker, Lanes 1-E: 16S gene amplicon of 20 *E. coli* isolates from clinical samples.

Table 1. GenBank accession numbers and β-cell lactamase assay of different *E. coli* strains under study.

Ser.	Bacterial strains	*β*-cell lactamase	Accession No.
1	*E. coli* strain WFGM S1	Positive	OR472881
2	*E. coli* strain WFGM S2	Positive	OR472882
3	*E. coli* strain WFGM S3	Positive	OR472883
4	*E. coli* strain WFGM S4	Positive	OR472884
5	*E. coli* strain WFGM S5	Positive	OR472885
6	*E. coli* strain WFGM S6	Positive	OR472886
7	*E. coli* strain WFGM S7	Positive	OR472887
8	*E. coli* strain WFGM S8	Positive	OR472888
9	*E. coli* strain WFGM S9	Positive	OR472889
10	*E. coli* strain WFGM S10	Positive	OR472890
11	*E. coli* strain WFGM S11	Positive	OR472891
12	*E. coli* strain WFGM S14	Positive	OR472892
13	*E. coli* strain WFGM S15	Positive	OR472893
14	*E. coli* strain WFGM S16	Positive	OR472894
15	*E. coli* strain WFGM S18	Positive	OR472895
16	*E. coli* strain WFGM S19	Positive	OR472896
1	*E. coli* strain WFGM S20	Positive	OR472897
18	*E. coli* strain WFGM S21	Positive	OR472898
19	*E. coli* strain WFGM S22	Positive	OR472899
Control	*E. coli* strain WFGM SE	Positive	OR472900

3.2. Antibiotic sensitivity assay of ESBL E. coli strains

In the present investigation, the beta-lactamase enzyme assay results indicated that all twenty studied *E. coli* strains produced beta-lactamase, and the positive result was observed as an inhibitory zone surrounding the bacterial colonies. The disc diffusion susceptibility test was carried out to determine the sensitivity or resistance of pathogenic microorganisms to various antimicrobial agents. On Mueller-Hinton agar, the pathogenic bacteria were cultivated in the presence of antimicrobial-impregnated filter paper discs. The presence or absence of bacterial growth around the discs is regarded an indirect sign of the compound's ability to inhibit the organism. Table 2 illustrates the antibiotic resistance characteristics of the pathogenic bacterial strains (Figure 3A, 3B). In order to evaluate the antibiotic resistance patterns of the examined bacterial strains, the Antibiot-

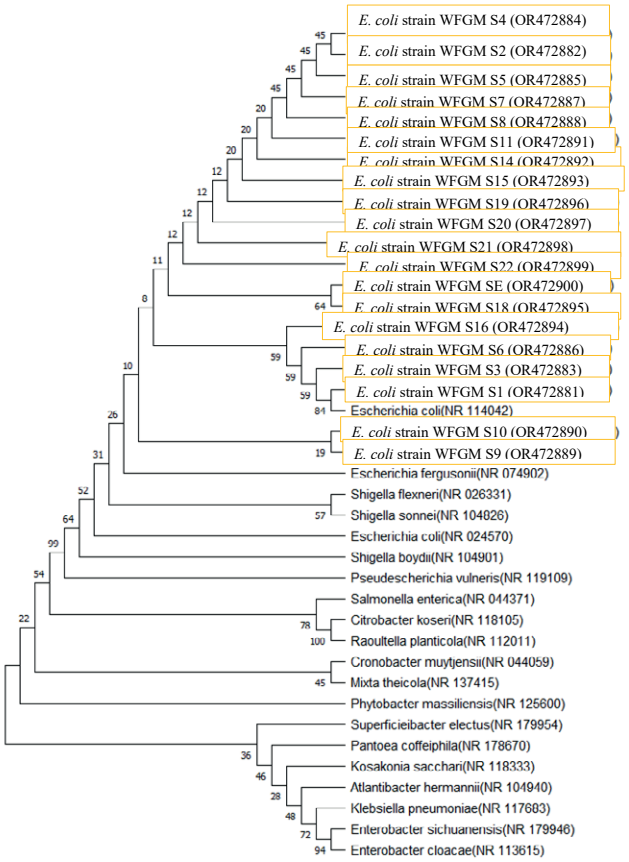

Figure 2. Phylogeny analysis of twenty *E. coli* strains from 16S region sequences compared with different related bacterial isolates obtained from GenBank.

ic-disk assay was developed utilising various antibiotics. The majority of the bacteria exhibited both antibiotic resistance and sensitivity, according to the results. Also, antibiotics susceptibility testing data revealed that the highest resistant pattern ranged between (44-61 percent) was found in 8 isolates (S11, S18, S3, S22, S7, S15, S16 and S20) from 19 (44 percent) examined bacteria, which were resistant to (9-11) antibiotics, followed by resistant pattern ranged between (39-44 percent) was observed in 7 isolates (S4, S5, S8, S14, S6, S14, S21, S6, S The resistance patterns of *E. coli* strains S9 and SE (control) are modest (5 percent and 6 percent, respectively). All examined *E. coli* isolates exhibited resistance to at least one of the antimicrobials examined. In particular, 95% of *E. coli* was resistant to CAZ, whereas 90% of same bacteria were resistant to AMP and CN. And 85% showed resistance to CXM and CFM. Eighty percent of the strains were resistant to AMC, and with a moderate susceptibility of 55%, 50% were resistant to CTX and COT, respectively. Additionally, 30% of the studied *E. coli* isolates were resistant to CIP, whereas 15% were resistant to CPZ. Only 10% of the examined *E. coli* bacteria were resistant to CTR and K, while 5% were resistant to GEN. None of the tested isolates were resistant to AK, NET, OF, or LE.

3.3. Antibacterial activity of pomegranate leaves and peels extracts

The antibacterial activity of pomegranate leaves and peels was evaluated against 20 *E. coli* isolates using the well diffusion technique. The results revealed significant heterogeneity in the zone of inhibition's size. To extract the active compounds from the powdered peel and leaves of pomegranate used in this investigation, two organic solvents (80% ethanol and 80% acetone) were employed. The antibacterial activity of the examined extracts in 2 percent DMSO was dose-dependent, ranging from 100 mg/ml to 500 mg/ml, and the inhibitory zone scale expanded as the concentration of the test extract increased.

The data in (Table 3) and (Figure 4) indicate that the ethanolic extracts 100-500mg/ml of pomegranate peels (EP) extract. Most the investigated strains showed resistance with absence of inhibition zone at 100mg/ml, compared with a strong antibacterial activity against the tested clinical *E. coli* strains, and showed maximum inhibition scale (9.0- 18.3 mm) as the EP extract concentration increased from 200 mg/ml to 500 mg/ml, followed by the inhibitory zone range (8.3-15.9 mm) at a dosage of 400 mg/ml EP extract, and inhibitory zone range (8.1-15.8 mm) at a dosage of 300 mg/ml EP extract. The more susceptible clinical *E. coli* strains to the (9.0- 18.3 mm) as the concentration increased from 200 mg/ml to 500 mg/ml EP extract were strains S16, S20, S3, S4, S6, S11, and S12 strains.

By regarding the antibacterial activity of acetone extraction of pomegranate peels (AP) at a dosage of 100-500 mg/ml as data represented in (Table 4) and (Figure 5). Most the investigated strains showed resistance with absence of inhibition zone at 100mg/ml and 200 mg/ml AP extract, and by increasing AP extract concentration from 200 mg/ml to 500 mg/ml demonstrated a potent antibacterial activity with a (9.07-15.3 mm) inhibitory zone ranges between tested clinical *E. coli* strains, followed by the inhibitory zone range (9.04-14.4 mm) at a dosage of 400 mg/ml AP extract, and inhibitory zone range (9.07-14.0 mm) at a dosage of 300 mg/ml AP extract. The more susceptible clinical *E. coli* strains to the (9.0-15.3 mm) as the concentration increased from 200 mg/ml to 500 mg/ml EP extract were strains S9, S20, S7, S8, and S10 strains.

Table 2. Resistance (%) or susceptibility of the ESBL *E. coli* strains used in the present study to different antibiotics

Strains	Resistance or inhibitory zone (mm) by antibiotics																		Antibiotic resistance (%)
	AMP	AK	AMC	CTX	CIP	CXM	CPZ	CAZ	GEN	NET	OF	NX	CFM	CTR	CN	K	LE	COT	
S1	7	21	R	11	25	22	10	9	17	23	23	20	R	12	R	18	26	29	22
S2	R	26	8*	30*	R	14*	27*	R	25*	16	14*	R	15*	28*	R	22*	22*	30*	28
S3	R	53	R	R	9	R	8	R	12	21	13	R	R	8	R	R	18	R	56
S4	R	37	R	10	R	R	14	R	11	21	11	14	R	16	R	13	16	31	39
S5	R	42	R	9	R	R	13	R	20*	19	13	R	R	13	R	14	18	25*	44
S6	R	42	R	R	25	R	11	R	19	23	25	20	R	9	R	19	27	R	44
S7	R	47	R	R	16*	R	9	R	17*	15	25	12*	R	8	R	R	30	R	50
S8	R	42	R	8	8	R	12	R	20	23	13	R	R	12	R	19	10	8	44
S9	11*	5	15*	29*	32	17*	28*	R	19*	22	32*	27*	16*	29*	17*	19*	30	31	6
S10	R	26	9	11	25	R	12	R	22*	20	24	19	R	15	R	18*	22	22	28
S11	R	58	R	R	R	R	8	R	12	20	8	R	R	R	R	15	8	R	61
S14	R	42	R	9	R	R	11	R	18	25	10	R	R	11	R	18	10	26	44
S15	R	47	R	R	20	R	R	R	17	21	21	18	R	9	R	17	25	R	50
S16	R	47	R	R	10	R	8	R	17	20	12	R	R	8	R	16	12	R	50
S18	R	58	R	R	R	R	R	R	19*	21	10	7	R	R	R	11	11	R	61
S19	R	37	R	R	25	R	8*	R	18	21	28	20	R	16	R	18	22	25	39
S20	R	47	R	R	22	R	R	R	17	21	25	24	R	8	R	17	25	R	50
S21	R	37	R	R	22	R	8	R	18	21	23	19	R	12	R	18	22	24	39
S22	R	53	R	R	9	R	9	R	R	17	8	R	R	9	R	15	11	R	56
SE	R	23	8	29	34	9	25	R	18	23	31	26	10	27	9	18	31	25	11

*R: Resistance. Antibiotic compounds used; AMP: Ampicillin, AK: Amikacin, AMC: Amoxyclav (Amoxycillin+Clavulinic Acid) (Augmentin), CTX: Cephotaxime, CIP: Ciprofloxacin, CXM: Cefuroxime, CPZ: Cefoperazone, CAZ: Ceftazidime, GEN: Gentamicin, NET: Netilmicin, OF: Ofloxacin, NX: Norfloxacin, CFM: Cefixime, CTR: Cefrtriaxone, CN: Cephalexin, K: Kanamycin, LE: Levofloxacin, COT: Co-Trimoxazole.

The antibacterial activity of ethanolic extraction of pomegranate leaves (EL) at a dosage of 100-500 mg/ml, and the bioactivity data against clinical *E. coli* strains represented in (Table 5) and (Figure 6). Most the investigated strains showed resistance, and no antibacterial activity was identified at 100mg/ml and 200 mg/ml EL extract, and by increasing EL extract concentration from 200 mg/ml to 500 mg/ml, the antibacterial activity significantly increased (8.5-12.6 mm) inhibitory zone ranges between tested clinical *E. coli* strains, followed by the inhibitory zone range (8.1-10.0 mm) at a dosage of 400 mg/ml EL extract, and inhibitory zone range (7.6-9.2mm) at a dosage of 300 mg/ml EL extract. The more susceptible clinical *E. coli* strains to the (8.0- 12.6 mm) as the concentration increased from 200 mg/ml to 500 mg/ml EL extract were strains S5, S2, S4, S7, S8, and S9 strains.

The antibacterial activity of 100-500 mg/ml acetone extraction of pomegranate leaves (AL) against clinical *E. coli* strains as demonstrated in (Table 6) and (Figure 7). The smallest concentrations (100mg/ml, 200mg/ml, 300 mg/ml AL extract showed high antibacterial resistance or a little antibacterial activity was identified, increasing AL extract concentration from 300 mg/ml to 500 mg/ml exhibited inhibitory zones spanning (7.8-10.2 mm) ranges between tested clinical *E. coli* strains, followed with inhibitory scale (8.0-9.5 mm) at a dosage of 400 mg/ml AL extract, and smallest inhibitory scale (7.8-8.1 mm) at a dosage of 300 mg/ml AL extract. The more susceptible clinical *E. coli* strains to the (8.7.8- 110.2mm) as the concentration increased from 200 mg/ml to 500 mg/ml AL extract were strains S6, S7, S8, S2, S6, and S17 strains.

4. DISCUSSION

Due to its numerous health benefits, *P. granatum* is not only a tasty fruit but also a therapeutic herb. Its leaves are abundant in phytochemicals with antibacterial characteristics (Stover and Mercure 2007; Fernández-Mazarrasa *et al*. 2009; Al-Sarraj 2021b). In this work, the researcher examined the antibacterial activity of pomegranate leaf extract against *E. coli* strains resistant to antibiotics. According to the data the inhibitory zone in different investigated bacterial strains were ranged

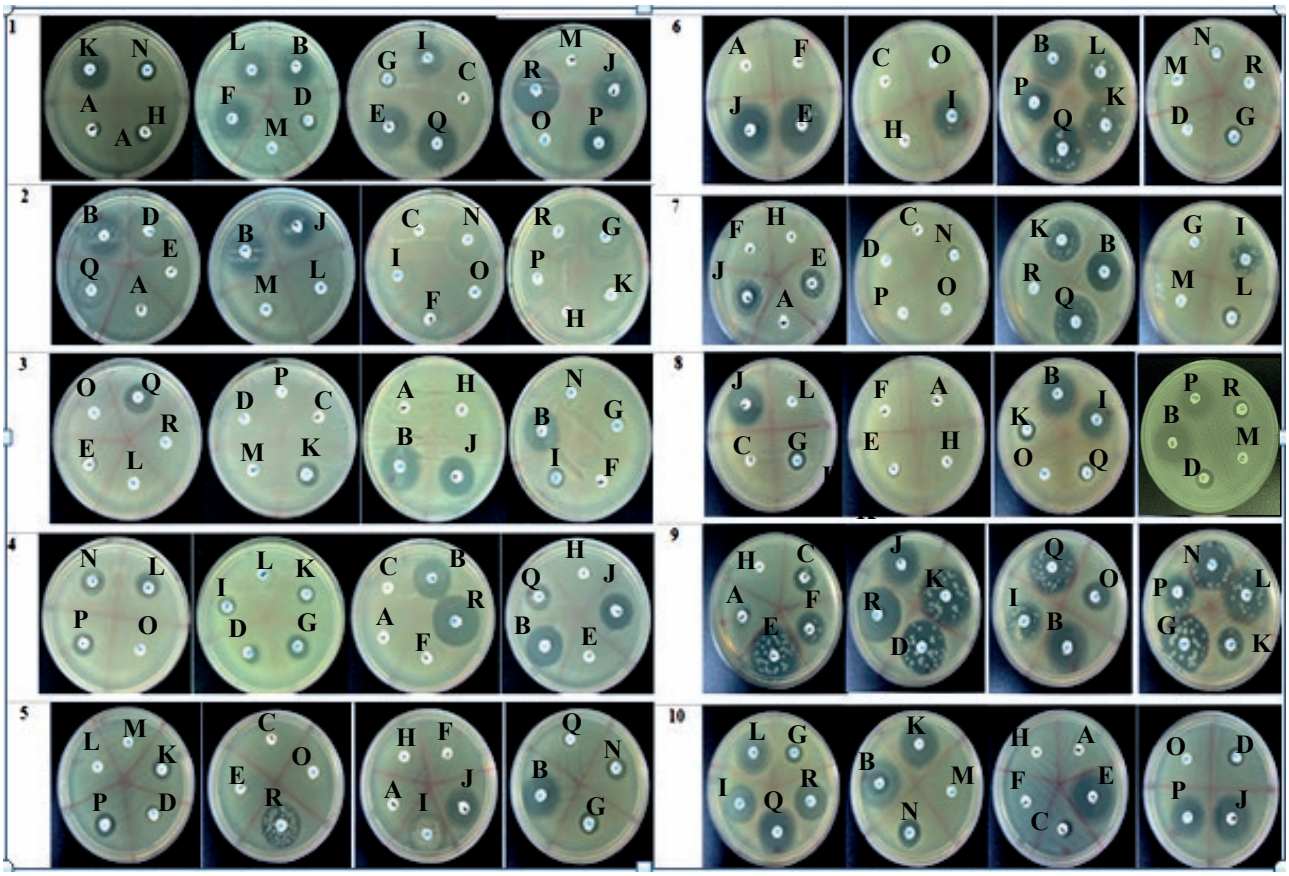

Figure 3A. Antibiotic resistance profiles of ten (1-10) *E. coli* pathogenic bacterial strains used in these studies. Antibiotic compounds used; Ampicillin (A), Amikacin (B), Amoxyclav (Amoxycillin+Clavulinic Acid) (Augmentin) (C), Cephotaxime (D), Ciprofloxacin (E), Cefuroxime (F), Cefoperazone (G), Ceftazidime (H), Gentamicin (I), Netilmicin (J), Ofloxacin (K), Norfloxacin (L), Cefixime (M), Cefrtriaxone (N), Cephalexin (O), Kanamycin (P), Levofloxacin (Q), Co-Trimoxazole (R).

between (7.6-18.3 mm). Concentration 200 mg/ml of ethanolic and acetone-extracted leaves resulted in formation a small scale of inhibition zone 7.6-8.5 mm) The 500 mg/ml concentration produced the greatest inhibition zone scale in ethanolic and acetone extraction of peels ranging between (9.0-18.3 mm), overall, at different concentrations (100 g/l - 500 mg/ml) pomegranate peel and leaves, the ethanol extracts of peel and leaves were more active than the acetone peel and leave extracts. The specifics are as follows. The leaf extract inhibited the proliferation and growth of *E. coli* that was resistant to antibiotics by breaking the cell membrane of the bacteria, so allowing antibiotics to enter the bacteria. The extract contains bioactive chemicals, including as tannins, alkaloids, and flavonoids, which generate antibacterial substances (Di Sotto *et al*., 2019; Alamshani *et al*., 2023; Zam and Khaddour 2017). The chemicals damage the bacterial cell membranes, leading in the release of the cellular contents and the eventual death of the bacterium. According to mass spectrometry research, *P. granatum* includes *oligomeric ellagitannins* consisting of up to 5 core glucose units, which are the most potent antibacterial chemicals in pomegranate.

In addition, multiple prior researches have demonstrated that the aqueous extract of pomegranate leaves inhibits the growth of uropathogenic *E. coli*. In addition, the extract exhibited a minimum inhibitory concentration (MIC) value of 0.6 mg/ml, resulting in an eighty percent reduction in the *E. coli* adhesion index. Additionally, the extract exhibited a minimum bactericidal concentration (MBC) of 1.2 mg/ml (Jam *et al*., 2022; Church *et al*., 2007). Thus, the peel extract inhibited *E. coli* biofilm formation and decreased bacterial adhesion capacity (Alamshani *et al*., 2023). Solvent extraction is a method used to extract antioxidant chemicals from plants. The research outcome is determined by the type and amount of solvent utilised during the extraction procedure (Ellatif *et al*., 2021; Ellatif *et al*., 2022a; Ellatif

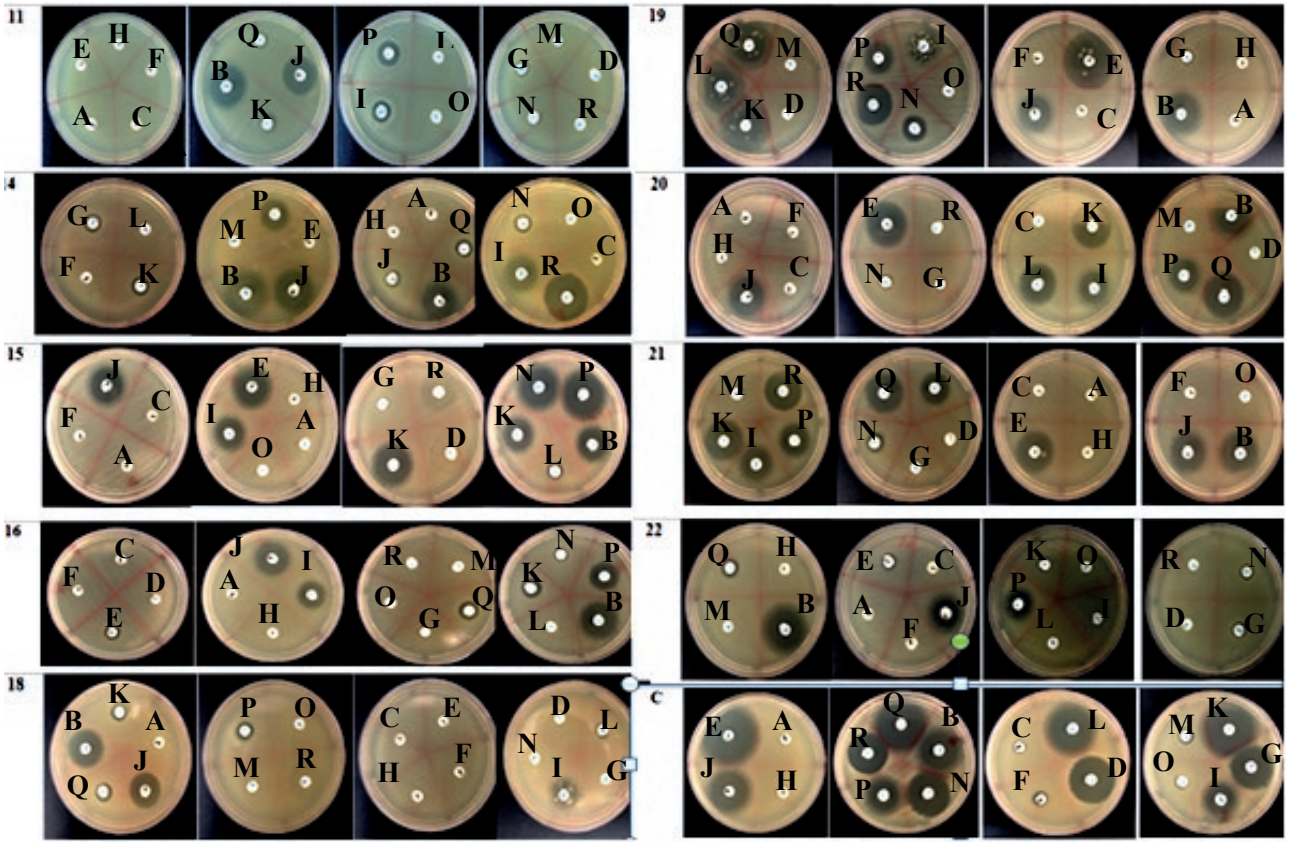

Figure 3B. Antibiotic resistance profiles of ten (11, 14, 15, 16, 18- 22 and C) *E. coli* pathogenic bacterial strains used in these studies. Antibiotic compounds used; Ampicillin (A), Amikacin (B), Amoxyclav (Amoxycillin+Clavulinic Acid) (Augmentin) (C), Cephotaxime (D), Ciprofloxacin (E), Cefuroxime (F), Cefoperazone (G), Ceftazidime (H), Gentamicin (I), Netilmicin (J), Ofloxacin (K), Norfloxacin (L), Cefixime (M), Cefrtriaxone (N), Cephalexin (O), Kanamycin (P), Levofloxacin (Q), Co-Trimoxazole (R).

et al., 2022b). For such experiments, aqueous solvents such as acetate, ethanol, ethyl acetate, and methanol are advised. Due to their polarity, ethanol and methanol have been widely utilised to extract antioxidant components from numerous plants and plant-based meals. Moreover, the two solvents are compatible with the human body (Sutan *et al*., 2023; Park *et al*., 2011). The plant was extracted using ethanol and acetone in this investigation. Mueller-Hinton agar is a regularly used solid culture medium for assessing the antibiotic susceptibility of microorganisms. Its composition (beef extract, casein hydrolysate, and starch) allows for precise testing of the susceptibility of microorganisms to antibiotics. Due to its low concentration of magnesium and calcium ions, Mueller-Hinton agar can be used for antimicrobial susceptibility testing. The low ion concentration inhibits the medium from reacting with certain antibiotics and guarantees that the medications' effects on the bacteria are not obscured. In this work, Mueller-Hinton agar was utilised to reveal *E. coli*'s susceptibility to the employed antibiotics. McConkey Agar is a differential and selective culture medium used to isolate and differentiate gram-negative bacteria, namely members of the Enterobacteriaceae family. It is composed of bile salts, neutral red indicators, crystal violet dye, peptones, and lactose (Erylmaz *et al*., 2010; Ho *et al*., These components promote the growth of Gram-negative bacteria while inhibiting the growth of Gram-positive bacteria. Its uniqueness rests on the capacity of bacteria to ferment lactose, hence creating acid and lowering the pH of the medium. This often results in a shift in the colour of the colonies, from pink to red (Phillips and Garda *et al*. 019; George *et al*., 2008; Giri *et al*., 2021). Effective culture medium because *E. coli* is an example of an enteric bacteria (Alamshani *et al*., 2023).

The results of the investigation reveal that the majority of strains demonstrated both antibiotic resistance and sensitivity. The most effective antibiotics against the pathogen *E. coli* are therefore Amikacin, Netilmicin, Ofloxacin, Cefrtriaxone, and Levofloxacin.

Table 3. The inhibition zone diameters (mm) of different concentrations (100-500 mg) of Pomegranate peels ethanol extracts against *E. coli* strains. Data are expressed as the mean ± SD

Strains	Pomegranate peels ethanol extracts (mg/ml)				
	100	200	300	400	500
1	R	R	8.6±0.01^{e}	14.6±0.12^{c}	16±0.24^{b}
2	R	R	14.3±0.21^{c}	15±0.14^{b}	15.8±0.13^{b}
3	R	9.1±0.12^{e}	14.1±0.12^{c}	15.90.12^{b}	17.2±0.21^{a}
4	R	R	13.2±0.11^{c}	15.7±0.14^{b}	17.1±0.15^{a}
5	R	8.2±0.14^{e}	9.6±0.04^{e}	10.3±0.1^{d}	11.6±0.02
6	R	R	9.3±0.025^{e}	10.2±0.012^{d}	11.1±0.11
7	R	R	8.1±0.014^{e}	10.3±0.14^{d}	12.3±0.14
8	9.0±0.02^{e}	11.3±0.13^{d}	13.3±0.11^{c}	14.6±0.012^{c}	15.6±0.12^{b}
9	R	R	R	8.4±0.02^{e}	10.0±0.01^{d}
10	R	10.6±0.11^{d}	12.1±0.07^{d}	13.6±0.04^{c}	14.6±0.1
11	R	R	12.2±0.10^{d}	14.05±0.12^{c}	15.2±0.09^{b}
12	R	8.0±0.01^{e}	10.1±0.011^{d}	14.3±0.13^{c}	15.6±0.14^{b}
13	R	R	R	R	R
14	R	R	R	R	R
15	R	R	R	R	9.0±0.05^{e}
16	R	R	15.8±0.12^{b}	14.2±0.011^{c}	18.3±0.24^{a}
17	R	R	R	R	9.2±0.10^{e}
18	R	10.1±0.05^{d}	11.2±0.1^{d}	12.02±0.10^{d}	13.3±0.12^{c}
19	R	R	R	8.3±0.04^{e}	9.1±0.01
20	R	R	9.1±0.05^{e}	12.3±0.013^{d}	16.1±0.13^{b}

*R: Resistance.

Table 4. The inhibition zone diameters (mm) of different concentrations (100-500 mg) of Pomegranate peels acetone extracts against *E. coli* strains. Data are expressed as the mean ± SD.

Strains	Pomegranate peels acetone extracts (mg/ml)				
	100	200	300	400	500
1	R	8.0±0.021^{d}	9.1±0.014^{d}	10.2±0.015^{c}	13.2±0.02^{b}
2	R	R	R	11.1±0.012^{c}	14.3±0.2^{a}
3	R	R	11.2±0.01^{c}	12.6±0.014^{b}	13.4±0.14^{b}
4	R	R	R	9.04±0.016^{d}	9.2±0.025^{d}
5	R	9.3±0.04^{d}	9.07±0.015^{d}	10.2±0.017^{c}	11.1±0.1^{c}
6	R	R	R	R	R
7	9.1±0.14^{d}	10.20.25^{c}	12.2±0.03^{b}	14.0±0.23^{a}	15.1±0.023^{a}
8	R	9.1±0.18^{d}	10.4±0.02^{c}	14.0±0.018^{a}	15.0±0.17^{a}
9	9.2±0.21^{d}	12.3±0.16^{b}	14.0±0.021^{a}	14.4±0.17^{a}	15.3±0.014^{a}
10	R	12.2±0.12^{b}	13.2±0.22^{b}	14.1±0.3^{a}	14.6±0.1^{a}
11	R	R	R	R	R
12	R	10.1±0.13^{c}	11.8±0.16^{c}	12.2±0.21^{b}	13.4±0.012
13	R	R	R	12.1±0.16^{b}	11.12±0.014^{c}
14	R	R	11.1±0.015^{c}	13.2±0.24^{b}	14.11±0.13^{a}
15	R	R	R	R	8.1±0.01^{d}
16	R	R	11.7±0.02^{c}	12.4±0.12^{b}	15.1±0.024^{a}
17	R	8.0±0.02^{d}	9.1±0.04^{d}	11.05±0.02^{c}	12.2±0.016^{b}
18	8.1±0.012^{d}	9.1±0.012^{d}	10.2±0.02^{c}	10.7±0.011^{c}	3±0.02^{b}
19	R	R	R	R	R
20	R	8.0±0.02^{d}	12.1±0.12^{b}	14.3±0.15^{a}	15.2±0.17^{a}

*R: Resistance.

These antibiotics were chosen because the drugs' microbial membrane was protected from damage by the bacteria injected. According to the findings, the pomegranate component extract has the greatest ability to inhibit the growth of pathogenic microorganisms (Alamshani *et al*., 2023). Additionally, the higher the concentration of the leaf extract, the simpler it is to alter the structural membrane of the *E. coli*-causing pathogen. This extract can be used to replace or even enhance the performance of standard antibiotics. Antimicrobial activity was greatest in the ethanolic extract. This is in contrast to acetone.

In conclusion, *P. granatum* leaves and peels have a substantial effect on *E. coli* that is resistant to antibiotics. The study indicated that the leaf extract had antibacterial properties that suppress the growth of *E. coli* strains resistant to antibiotics. The active chemicals in the extract eliminate antibiotic resistance by destroying the integrity of the bacteria. When *P. granatum* leaf extract is mixed with standard antibiotics, synergistic effects are produced. The combo eradicates antibiotic-resistant *E. coli* bacteria more effectively than conventional antibiotics alone. These findings demonstrate the potential of *P. granatum* leaves as a natural alternative and treatment for conventional antibiotics in the management and treatment of antibiotic-resistant bacterial illnesses. To determine the precise mechanisms of action, optimise the extraction procedures, and assess the long-term efficacy and safety of *P. granatum* leaf extracts, additional research is required. However, the antibacterial properties of *P. granatum* have permitted the development of innovative, safe, and successful therapeutic techniques against *E. coli* resistant to antibiotics. tannins and alkaloids metabolites in the leaves and peels of pomegranate are considered antimicrobial (Joshi *et al*., 2019; Shaygannia *et al*. 2016; Wu and Tian 2017; Warsi and Sulistyani 2018; Joshi and Nair 1960). Alkaline groups in alkaloids interact with amino acid groups in cells, resulting in structural and chemical changes that are damaging to the cell (Johan *et al*., 2020; Seeram *et al*., 2005; Nurdin *et al*., 2019). Antibacterial flavonoids in pomegranate inhibit bacterial growth by inhibiting the DNA gyrase mechanism, resulting in intracellular leakage (Shaygannia *et al*., 2016). Queercetin, a flavonoid found in pomegranate, may kill microorganisms by increasing membrane permeability and hurting the potential of bacteria within membranes (Khan *et al*. 2012).

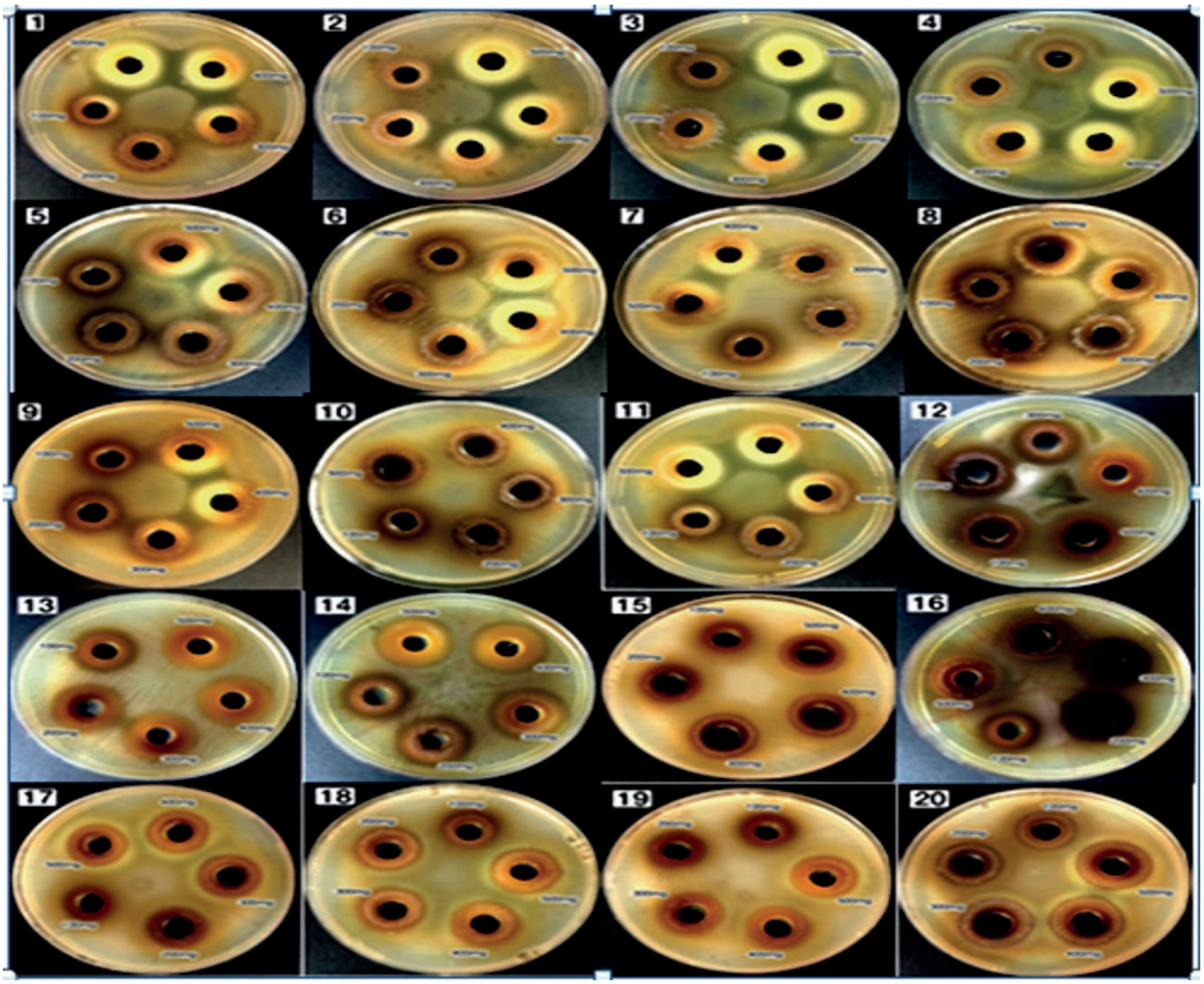

Figure 4. The inhibition zone diameters of different concentrations (100mg-500mg) of Pomegranate peels ethanol extracts against *E. coli* strains.

5. CONCLUSION

As a result of the global problem with MDR bacteria, scientists have been attempting to develop novel bioactive compounds derived from natural resources that can be exploited as safe phytotherapy. According to the findings of this study, pomegranate peels and leaf extract can suppress the growth of Extended-Spectrum Beta-Lactamase -*E. coli* pathogenic strains at doses 100 and 200 mg/ml with highest significant antibacterial activity. Accordingly, the ethanolic and acetone extracts exhibited the best antibacterial activity and may be used to enhance or even replace antibiotics with less cytotoxic effect.

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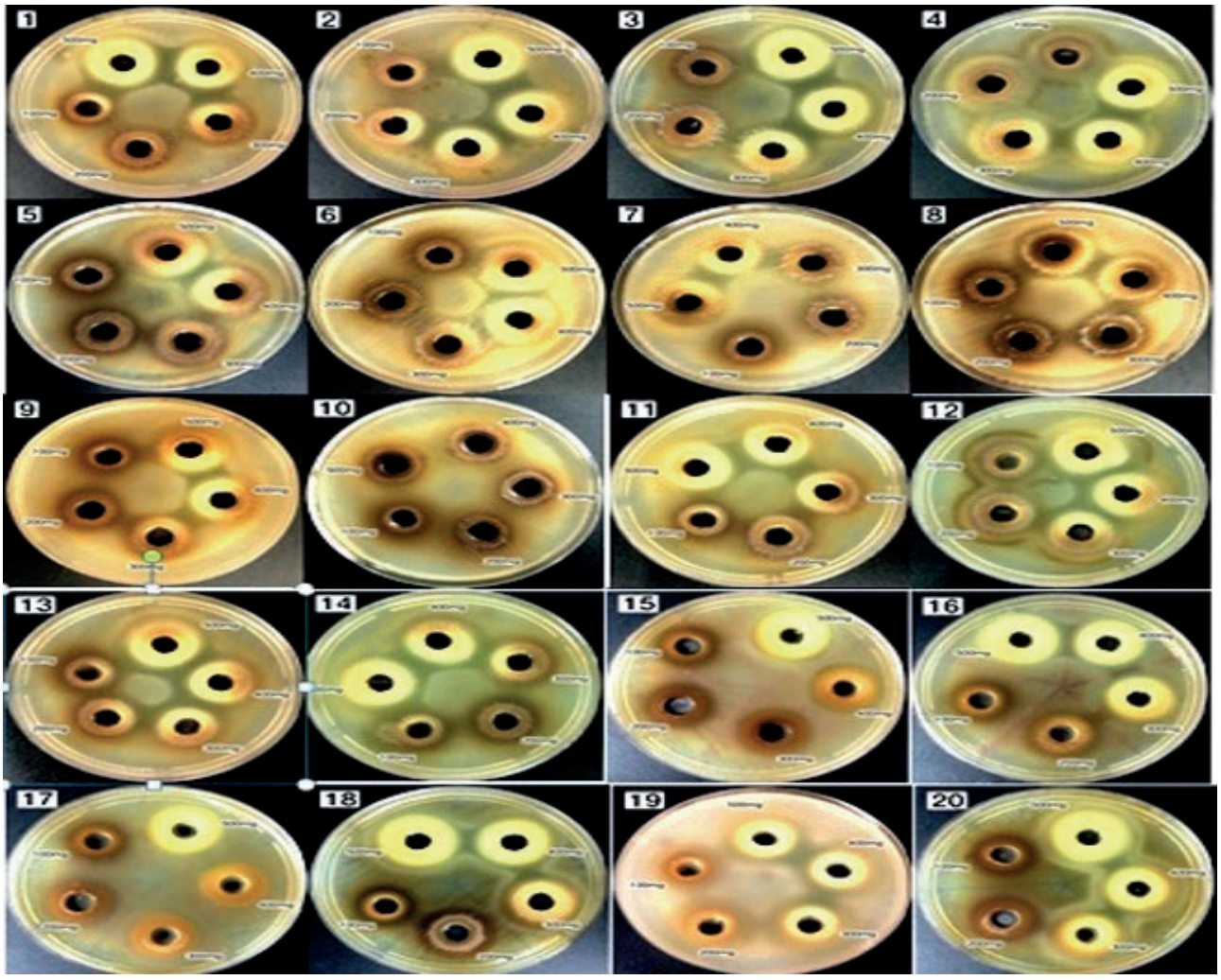

Figure 5. The inhibition zone diameters of different concentrations (100-500 mg) of Pomegranate peels acetone extracts against *E. coli* strains.

AUTHOR CONTRIBUTIONS

All the authors contributed to the study's conception and design. The Faisal Al-Sarraj, Raed Albiheyri and Noor M. Bataweel authors were Identify ultimate objective of study and designed the required analysis and bio-assays. Wafa H. Alamshani, Majid Al-Zahrani, Tahani M. Alqahtani, Mashail A. Alghamdi, Nada Nass and Thamer Bouback authors were involved in funding acquisition, prepare the chemical, kits and performed the practical part of this work. Ibrahim Alotibi, Mohammed A. Al-Matary and Bayan H. Sajer authors were participate in study analysis, obtained raw data collection, supervision, and perform the statistical analysis. Faisal Al-Sarraj, Raed Albi, M. Attallah and Wafa H. Alamshani authors were shared in paper writing the manuscript body, create a strong conclusion and language editing

REFERENCES

Alamshani HW, Al-Sarraj FM, Algamdi M. 2023. The inhibitory effect of *Punica granatum* on *Escherichia coli* and *Klebsiella pneumonia* extended spectrum β-lactamase strains. Novel Research in Microbiology Journal, 7(1):1836-56.

Alotibi I, Al-Sarraj FM, Albiheyri R, Alghamdi MA, Nass N, Bouback T, Sajer BH, Al-Zahrani M, Basingab F, Alharbi M. 2022. Study the apoptosis and necrosis inducing of fosfomycin into associated infected urothelial tissue by extended spectrum beta lactamase positive of *E. coli*. Microbial Pathogenesis. 173:105838.

Al-Sarraj FM. 2021a. The effect of antibiotics and photodynamic therapy on *extended-spectrum beta-lactamase (ESBL) positive of Escherichia coli and Klebsiella*

Table 5. The inhibition zone diameters (mm) of different concentrations (100-500 mg) of Pomegranate leaves ethanol extracts (EL) against *E. coli* strains. Data are expressed as the mean ± SD.

Strains	Pomegranate peels acetone extracts (mg/ml)				
	100	200	300	400	500
1	R	R	R	R	8.0±0.011^{d}
2	R	8.0±0.010^{d}	9.0±0.1^{c}	10.0±0.02^{b}	11.6±0.04^{a}
3	R	R	7.8±0.03^{d}	8.0±0.011	8.4±0.012^{d}
4	7.6±0.01^{d}	8.0±0.03^{d}	8.6±0.07^{d}	9.3±0.012	10.0±0.03^{b}
5	8.0±0.02^{d}	8.5±0.013^{d}	9.2±0.02^{c}	9.6±0.011	10.0±0.02^{b}
6	R	R	R	8.1±0.015^{c}	8.7±0.012^{d}
7	R	R	8.7±0.01^{c}	9.8±0.2^{c}	12.6±0.025^{a}
8	R	R	8.0±0.02^{d}	9.3±0.1^{c}	12.0±0.017^{a}
9	R	R	7.6±0.02^{d}	R	8.5±0.011^{d}
10	R	R	R	R	9.0±0.013^{c}
11	R	R	R	R	R
12	R	R	8.3±0.011^{c}	8.6±0.012^{d}	9.0±0.014^{c}
13	R	R	R	R	R
14	R	R	R	R	R
15	R	R	R	R	R
16	R	R	R	R	R
17	R	R	R	8	R
18	R	R	R	R	R
19	R	R	R	R	R
20	R	R	R	R	R

*R: Resistance.

Table 6. The inhibition zone diameters (mm) of different concentrations (100-500 mg) of Pomegranate leaves acetone extracts (AL) against *E. coli* strains. Data are expressed as the mean ± SD.

Strains	Pomegranate peels acetone extracts (mg/ml)				
	100	200	300	400	500
1	R	R	R	R	R
2	R	7.6±0.012^{c}	8.1±0.02^{c}	8.4±0.015^{c}	9.2±0.014^{b}
3	R	R	R	R	R
4	R	R	R	R	R
5	R	R	R	R	8.1±0.011^{a}
6	R	R	8.0±0.014^{c}	9.0±0.025^{b}	10.1±0.12^{a}
7	R	R	R	9.5±0.04^{b}	10.2±0.015^{a}
8	R	R	8.0±0.012^{c}	9.3±0.12^{b}	10.1±0.21^{a}
9	R	R	R	R	9.2±0.013^{b}
10	R	R	R	R	8.0±0.01^{c}
11	R	R	R	R	R
12	R	R	8	8	8.0±0.013^{c}
13	R	R	R	R	8.0±0.01^{c}
14	R	R	R	R	8.1±0.012^{c}
15	R	R	R	R	R
16	R	R	R	R	R
17	R	R	7.8±0.011^{c}	8.0±0.012^{c}	8.6±0.011
18	R	R	R	R	8.0±0.01^{c}
19	R	R	R	R	8.1±0.12^{c}
20	R	R	R	R	R

*R: Resistance.

pneumoniae in urothelial cells. Saudi J Biol Sci.28: (10):5561-7.

Al-Sarraj FM. 2021b. A Review on the impacts of *Azadirachta indica* on Multi-drug Resistant Extended Spectrum Beta Lactamase-positive of *Escherichia coli* and *Klebsiella pneumonia*. Adv Life Sci. 8(3):228-32.

Amanulla AM, Sundaram RJ. 2019. Green synthesis of TiO_2 nanoparticles using orange peel extract for antibacterial, cytotoxicity and humidity sensor applications. Materials Today: Proceedings. 8:323-31.

Azcárate-Peril, M. A., & Raya, R. R. 2001. Methods for plasmid and genomic DNA isolation from l*actobacilli*. Food microbiology protocols. 135-139

Berthe T, Ratajczak M, Clermont O, Denamur E, Petit F. 2013. Evidence for coexistence of distinct *Escherichia coli* populations in various aquatic environments and their survival in estuary water. Appl environ microbiol, 79(15):4684-93.

Chauhan A, Jindal T, Chauhan A, Jindal T. 2020. Microbiological Culture Media: Types, Role and Composition. Microbiological Methods for Environment, Food and Pharmaceutical Analysis.23-66.

Church DL, Emshey D, Semeniuk H, Lloyd T, Pitout JD. 2007. Evaluation of BBL CHROMagar O157 versus Sorbitol-MacConkey medium for routine detection of *Escherichia coli* O157 in a centralised regional clinical microbiology laboratory. J Clin Microbiol. 45(9):3098-100.

Denamur E, Clermont O, Bonacorsi S, Gordon D. 2021. The population genetics of pathogenic *Escherichia coli*. Nature Reviews Microbiology. 19(1):37-54.

Di Sotto A, Locatelli M, Macone A, Toniolo C, Cesa S, Carradori S, Eufemi M, Mazzanti G, Di Giacomo S. 2019. Hypoglycemic, antiglycation, and cytoprotective properties of a phenol-rich extract from waste peel of *Punica granatum* L. var. Dente di Cavallo DC2. Molecules. 24(17)

Eryılmaz M, Bozkurt ME, Yildiz MM, Akin A. 2010. Antimicrobial resistance of urinary *Escherichia coli* isolates. Tropical Journal of Pharmaceutical Research, 9(2):205-209.

Facciola, Stephen. "Cornucopia—A Source Book of Edible Plants," Kampong Publications, Vista, 1990. 677 pp. Paperback. (LC 90-92097, ISBN 0-9628087-0-9).

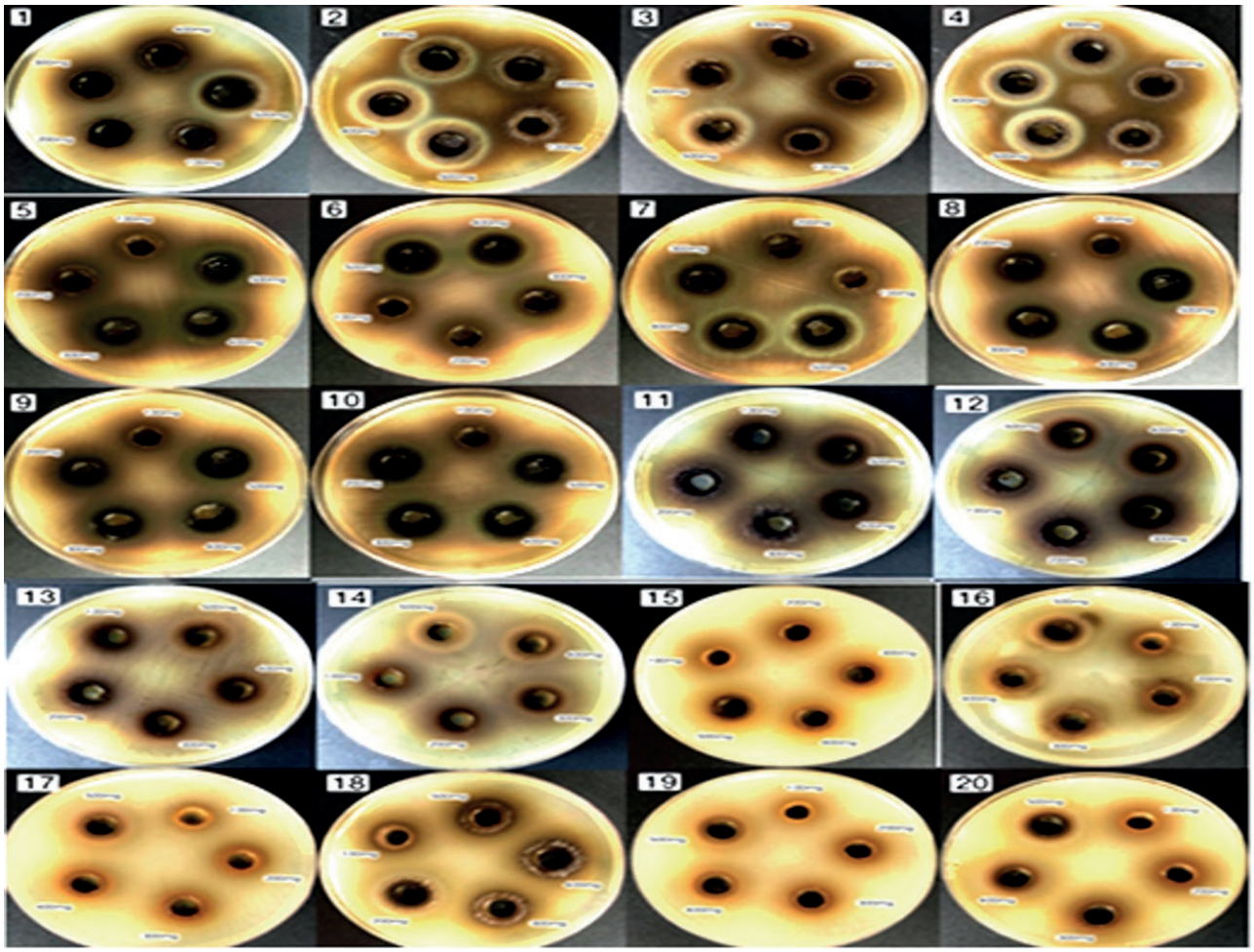

Figure 6. The inhibition zone diameters of different concentrations (100-500 mg) of Pomegranate leaves ethanol extracts against *E. coli* strains.

Fernández-Mazarrasa C, Mazarrasa O, Calvo J, del Arco A, Martínez-Martínez L. 2009. High concentrations of manganese in Mueller-Hinton agar increase MICs of tigecycline determined by Etest. Journal of Clinical Microbiology 47(3):827-9. https://doi.org/10.1128/JCM.02464-08. Epub 2009 Jan 14. PMID: 19144806; PMCID: PMC2650928.

Flores-Mireles AL, Walker JN, Caparon M, Hultgren SJ. 2015. Urinary tract infections: epidemiology, mechanisms of infection and treatment options. Nat Rev Microbiol. 13(5):269-284.

George EF, Hall MA, Klerk GJ. 2008. Plant tissue culture procedure-background. Plant Propagation by Tissue Culture: Volume 1. The Background. 1-28.

Giri N, Lodhi A, Bisht DS, Bhoj S, Arya DK. 2021. Antibiotic Resistance of *Escherichia Coli* Isolated from Lake Nainital, Uttarakhand State, India. J Mountain Res. 16:127-36.

Hashemi SH, Hashemi N, Esna-Ashari F, Taher A, Dehghan A. 2017. Clinical features and antimicrobial resistance of bacterial agents of ventilator-associated tracheobronchitis in Hamedan, Iran. Oman Med J., 32(5):403-408.

Hashim I, Pharma S. 2013. Microbiological culture media in pharmaceutical industry. Foster city, *USA:* OMICS Group eBooks.

Heidari H, Hasanpour S, Ebrahim-Saraie HS, Motamedifar M. 2017. High incidence of virulence factors among clinical *enterococcus faecalis* isolates in Southwestern Iran. Infect Chemother, 49(1):51-56.

Ho PL, Chow KH, Yuen KY, Ng WS, Chau PY. 1998. Comparison of a novel, inhibitor-potentiated disc-diffusion test with other methods for the detection of extended-spectrum beta-lactamases in *Escherichia coli* and *Klebsiella pneumoniae*. J Antimicrob Chemother 42(1):49-54.

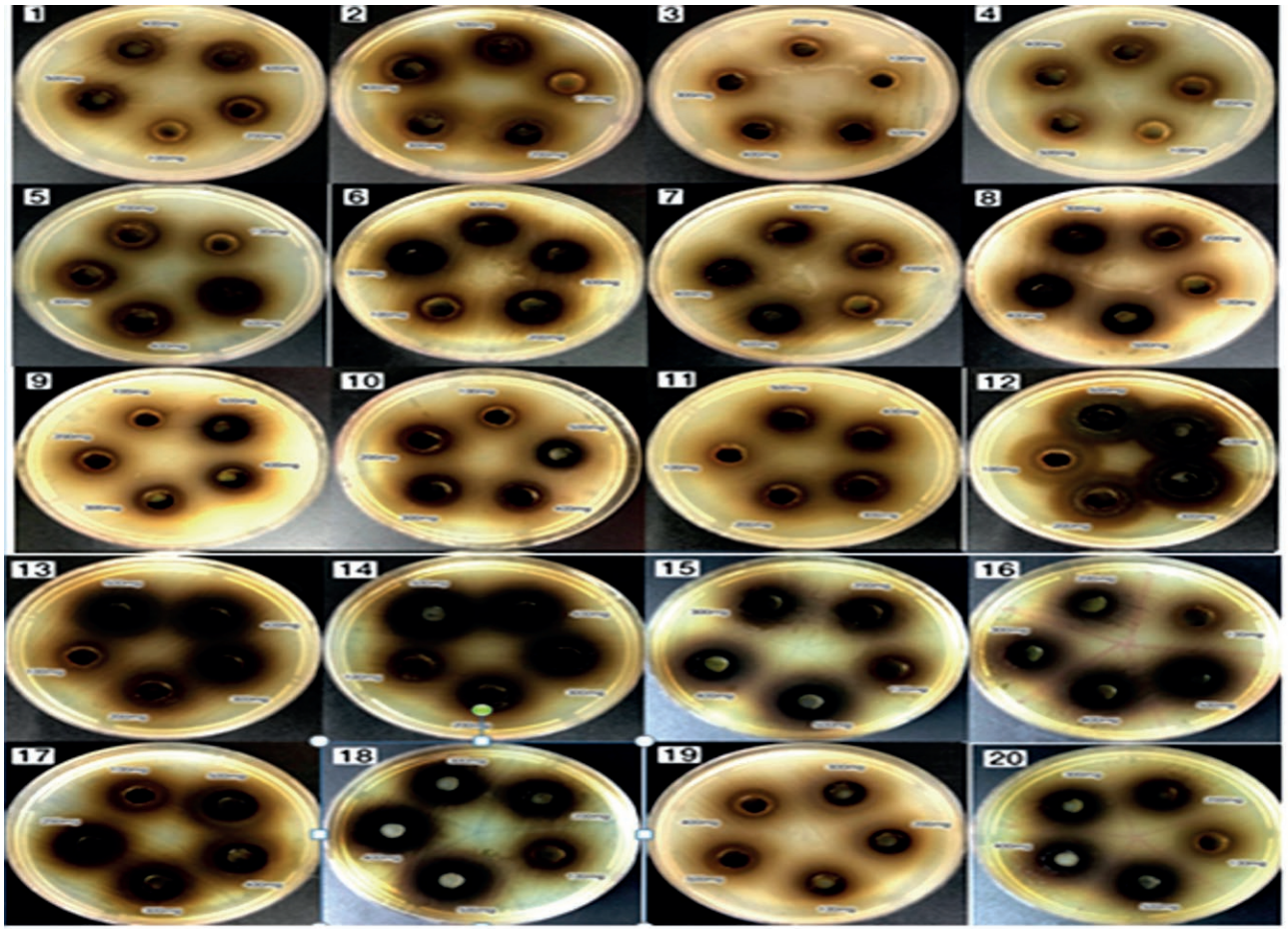

Figure 7. The inhibition zone diameters of different concentrations (100-500 mg) of Pomegranate leaves acetone extracts against *E. coli* strains.

Houri H, Kazemian H, Sedigh Ebrahim-Saraie H, Taji A, Tayebi Z, Heidari H. 2017. Linezolid activity against clinical Gram-positive cocci with advanced antimicrobial drug resistance in Iran. J Glob Antimicrob Resist. 10:200-203.

Huang Z, Peng J, Han J, Zhang G, Huang Y, Duan M, Liu D, Xiong Y, Xia S, Lai W. 2019. A novel method based on fluorescent magnetic nanobeads for rapid detection of Escherichia coli O157: H7. Food chemistry. 276:333-41.

Jam N, Hajimohammadi R, Gharbani P, Mehrizad A. 2022. Antibacterial activity of *Punica granatum* L. and Areca nut (PA) combined extracts against some food born pathogenic bacteria. Saudi J Biol Sci. 29(3):1730-6.

Johan A, Natalia A, Djauhari W, Effendi RF. 2017. Clinical and hemoglobin profile of malaria patients in Karitas Hospital, Southwest Sumba, and period of year. Indones J Trop Infect Dis. 8(1):1. https://doi.org/10.20473/ijtid.v8i1.11455

Joshi C, Patel P, Kothari V. 2019. Anti-infective potential of hydroalcoholic extract of *Punica granatum* peel against gram-negative bacterial pathogens. *F1000Research* 8:70. https://doi.org/10.12688/f1000research.17430.1

Joshi G V., Nair KK. 1960. Journal of Biological Sciences. J Ecol., 48(3):752. https://doi.org/10.2307/2257356

Kam AH, Neergheen VS. 2022. Nutraceutical and phytopharmaceuticals in immune health. Innutrition and Functional Foods in Boosting Digestion, Metabolism and Immune Health, (pp. 445-475). Academic Press.

Kazemian H, Heidari H, Ghanavati R, Mohebi R, Ghafourian S, Shavalipour A, et al. 2016. Characterization of antimicrobial resistance pattern and molecular analysis among extended spectrum β-lactamase-producing *Escherichia coli*. Pharm Sci. 22(4):279-284.

Khalid M, Andreoli S. 2019. Extrarenal manifestations of the haemolytic uremic syndrome associated with

Shiga toxin-producing *Escherichia coli* (STEC HUS). Paediatric Nephrology, 34:2495-507.

Khan N, Syed DN, Pal HC, Mukhtar H, Afaq F. 2012. Pomegranate fruit extract inhibits UVB-induced inflammation and proliferation by modulating NF-κB and MAPK signaling pathways in mouse skin. Photochem Photobiol. 88(5):1126-1134. https://doi.org/10.1111/j.1751-1097.2011.01063.x

Kim YH, Choi EM. 2009. Stimulation of osteoblastic differentiation and inhibition of interleukin-6 and nitric oxide in MC3T3-E1 cells by pomegranate ethanol extract. Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives. 23(5):737-9.

Kumar S., Stecher G., Li M., Knyaz C., and Tamura K. 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Mol Biol Evol 35:1547-1549.

Kunle, Oluyemisi Folashade, Egharevba, Henry Omoregie and Ahmadu, Peter Ochogu. 2012. Standardization of herbal medicines - A review. Int. J. Biodivers. Conserv. 4(3), pp. 101-112

Lateef A., Oloke J. K., Gueguim-Kana E. B. 2004. Antimicrobial resistance of bacterial strains isolated from orange juice products. Afr J Biotechnol. 3(6):334-338. https://doi.org/10.5897/ajb2004.000-2061

Loizzo MR, Aiello F, Tenuta MC, Leporini M, Falco T, Tundis R. 2019. Pomegranate (*Punica granatum* L.). In Non vitamin and non mineral nutritional supplements Jan 1 (pp. 467-472). Academic Press.

Motamedifar M, Ebrahim-Saraie HS, Abadi AR, Moghadam MN. 2015. First outcome of MDR-TB among Co- Infected HIV/TB patients from South-West Iran. Tuberc Respir Dis (Seoul). 78(3):253-257.

Nurdin KE, Olla LRY, Feoh SF, et al. 2019. Effectively Test of 96% from Soe (*Citrus sinensis* L.) Sweet Orange Rind Ethanol Extract as Biolarvaside. J Info Kesehat. 17(2):176-183. https://doi.org/10.31965/infokes.17(2):278.

Park SH, Ryu S, Kang DH. 2011. Improved selective and differential medium for isolation of *Escherichia coli* O157: H7. J Clinic Microb. 49(1):405-8.

Phillips GC, Garda M. 2019. Plant tissue culture media and practices: an overview. In Vitro Cellular & Developmental Biology-Plant 55: 242-57.

Pourakbari B, Ferdosian F, Mahmoudi S, Teymuri M, Sabouni F, Heydari H, et al. 2012. Increase resistant rates and ESBLproduction between *E. coli* isolates causing urinary tract infection in young patients from Iran. Braz J Microbiol. 43(2):766-769.

Qi Z, Li G, Wang M, Chen C, Xu Z, An T. 2022. Photoelectrocatalytic inactivation mechanism of *E. coli* DH5α (TET) and synergistic degradation of corresponding antibiotics in water. Water Research. 215:118240.

Ricci D, Giamperi L, Bucchini A, Fraternale D. 2006. Antioxidant activity of *Punica granatum* fruits. Fitoterapia. 77(4):310-2.

Ellatif, SA, Nermine NA, Amira YM , Enayat MD, and Dalia KA 2021. Production, purification and identification of glycine,N-(m-anisoyl)-methyl ester from *Pseudomonas aeruginosa* with antimicrobial and anticancer activities. Kuwait j of science.48(3):1-12.

Ellatif, SA, Bouqellah, NA, Abu-Serie, MM. et al. 2022a. Assessment of probiotic efficacy and anticancer activities of *Lactiplantibacillus plantarum* ESSG1 (MZ683194.1) and *Lactiplantibacillus pentosus* ESSG2 (MZ683195.1) isolated from dairy products. Environ Sci Pollut Res.29 (26):39684-39701 https://doi.org/10.1007/s11356-022-18537-z

Ellatif, SA., Abdel Razik, ES, Abu-Serie, M.M, Mahfouz A, Shater AF, Saleh FM, Hassan MM, Alsanie WF, Altalhi A, Daigham GE, et al. 2022b. Immunomodulatory Efficacy-Mediated Anti-HCV and Anti-HBV Potential of Kefir Grains; Unveiling the In Vitro Antibacterial, Antifungal, and Wound Healing Activities. Molecules 2022, 27, 2016. https://doi.org/10.3390/molecules27062016

Seeram NP, Adams LS, Henning SM, et al. 2005. In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. J Nutr Biochem. 16(6):360-367. https://doi.org/10.1016/j.jnutbio.2005.01.006

Shaikh S, Fatima J, Shakil S, Rizvi SM, Kamal MA. 2015. Antibiotic resistance and extended spectrum beta-lactamases: Types, epidemiology and treatment. Saudi J Biol Sci. 22(1):90-101.

Shaygannia E, Bahmani M, Zamanzad B, Rafieian-Kopaei M. 2016. A Review Study on *Punica granatum* L. J Evid Based Complementary Altern Med. 21(3):221-227. https://doi.org/10.1177/2156587215598039

Sneath P.H.A. and Sokal R.R. 1973. *Numerical Taxonomy*. Freeman, San Francisco. Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.

Şuţan NA, Bărbuceanu M, Bărbuceanu D, and Deliu I 2023. Cytogenotoxic and antimicrobial effects of *Nezara viridula* (L.) (Hemip-tera: Heteroptera: Pentatomidae) alcoholic extracts. Caryologia 76(4): 39-49. https://doi.org/10.36253/caryologia-2389

Stover ED, Mercure EW. 2007. The pomegranate: a new look at the fruit of paradise. Hort Science. 42(5):1088-92.

Tamura K., Nei M., and Kumar S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences (USA) 101:11030-11035.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molec Biol Evol 28(10):2731-2739.

Warsi W, Sulistyani N. 2018. The Optimization of Secondary Metabolite Production Time and Screening Antibacterial Activity of Actinomycetes Isolate from Tin Plant Rizosfer (*Ficus carica*). J Teknol Lab. 7(1):15. https://doi.org/10.29238/teknolabjournal.v7i1.120

Wu S, Tian L. 2017. Diverse Phytochemicals and Bioactivities in the Ancient Fruit and Modern Functional Food Pomegranate (*Punica granatum*). Molecules. 22(10):1606. https://doi.org/10.3390/molecules22101606

Zam W, Khaddour A. 2017. Anti-virulence effects of aqueous pomegranate peel extract on *E. coli* urinary tract infection. Progr Nutr. 19:98-104.

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