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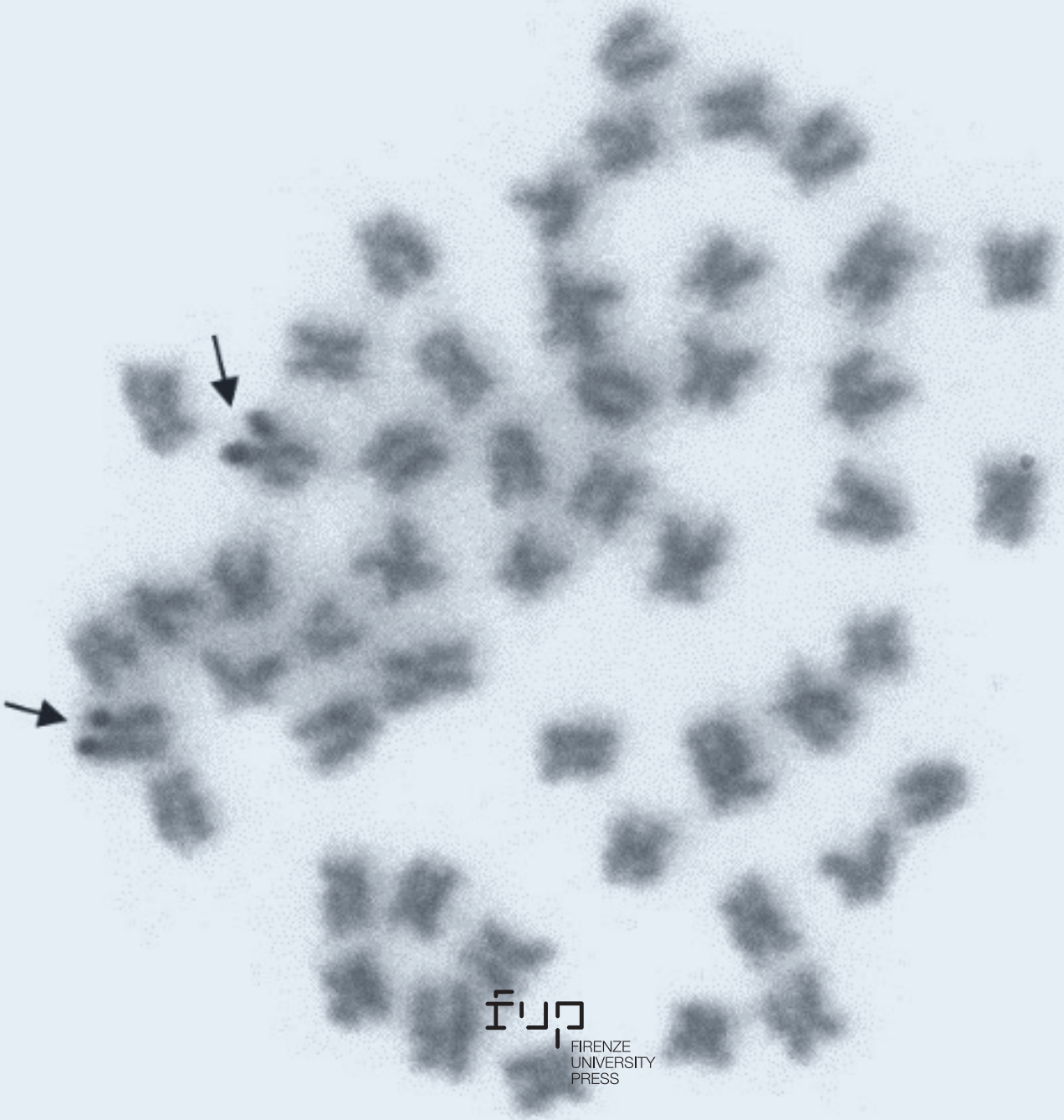

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First cytogenetic study of the Somphong's rasbora (*Trigonostigma somphongsi*) (Perciformes, Cyprinidae), a critically endangered species in Thailand

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Abstract. The first chromosome study of *Trigonostigma somphongsi* (Meinken,1958) from Thailand. Specimens were collected from Pak Phli District, Nakhornnayok Province, central Thailand. The mitotic chromosomes were directly prepared from kidney tissues of two males and two females. Conventional staining and Ag-NOR banding techniques were applied to stain the chromosomes. The results shown that diploid chromosome number of *T. somphongsi* was 2*n*=50, the fundamental numbers (NF) were 92 in both male and female. The types chromosomes consisted of 10 metacentric, 20 submetacentric, 12 acrocentric and 8 telocentric chromosomes. The result also exhibited that the interstitial nucleolar organizer regions (NORs) were clearly observed at the short arm of chromosome pair 2. The karyotype formula could be deduced as: 2*n* (diploid) 50=10m+20sm+12a+8t.

Keywords: karyotype, chromosome, *Trigonostigma somphongsi.*

INTRODUCTION

The *Trigonostigma somphongsi* is a critically endangered species in natural habitat of Thailand. It has been discovered in a deep water rice field, floodplain of Bangpakong Basin, Nakhornnayok Province, central Thailand. (Petsut et al. 2014). The *Trigonostigma* is a genus of small cyprinid fish found in Southeast Asia. There are currently five recognized species in this genus including *T. heteromorpha*, *T. hengeli*, *T. somphongsi*, *T. espei* and *T. truncate* (Tan, 2020). The *T. somphongsi* was found in the rice field and in a

Figure 1. The characteristic of *T. somphongsi*. Scale bar indicate 0.2 cm.

densely vegetated ditch, which was flooded by a nearby river. It differs from the rest of the genus in its dark pattern which occurs as a strait horizontal line that extends from the base of the caudal fin and ends just after it passed the posterior part of the dorsal fin, instead of showing the wedge-like marking of other species in the genus (Petsut et al, 2014) (Fig. 1). Although the Somphong's rasbora is important for fish biodiversity of Thailand, there were quite scarce data about cytogenetics in these fishes especially banding analysis in fish chromosomes.

Up to date, information about karyotypes in genus *Trigonostigma* are rare and usually based on conventional staining technique. Only *T. heteromorpha* has been published, and the result showed that the diploid chromosome number (2*n*) was 50 (Khuda 1979). The cytogenetic studies using conventional staining technique provided valuable information on the great karyotype diversity shown by these animals. Analyses of cytogenetic markers, included the number and karyotype formula, number and location of nucleolar organizer regions (NORs). The present study is the first report on the chromosomal characteristics of *T. somphongsi* determined using conventional staining and Ag-NOR banding techniques. *T. somphongsi* is a critically endangered species in natural habitats of Thailand. The results enhance the level of cytogenetic information available and enable future comprehensive studies to be conducted on taxonomy and evolutionary relationships. Moreover, the data provide useful basic information for conservation and on breeding practices as well as an analysis of the chromosomal evolution of this species of *Trigonostigma*.

MATERIALS AND METHODS

Sample collection

Two males and two female of *T. somphongsi* were collected from agricultural land, Pak Phli District, Nakhornnayok Province, central Thailand, and were grown starting from May 2023 (permission from an ethical committee ID U1-04498-2559). All specimens were maintained in aerated, flowing seawater acquaria until the analysis.

Chromosome preparation

Chromosomes preparations were obtained from kidney by cell suspension technique. Briefly, the kidney was cut into small pieces and then mixed with 0.075 M potassium chloride (KCl). After discarding all large piece tissues, cell sediments were transferred to a centrifuge tube and incubated for 30 minutes, then centrifuged at 1,500 rpm for 5 minutes. The KCl was discarded from the supernatant after centrifugation at 1,500 rpm for 5 minutes. Cells were fixed in fresh cool fixative (3 methanol:1 glacial acetic acid) and gradually made up to 8 ml before centrifuging again at 1,500 rpm for 5 minutes, whereupon the supernatant was discarded. Fixation was repeated until the supernatant was clear. The mixture was dropped onto a clean and cold slide by micropipette followed by air-drying technique.

Chromosome staining

Conventional staining of the chromosomes in the air-dried slides was done using 10% Giemsa solution for 10 minute (Rooney 2001). Ag-NOR banding was carried out following method of Howell and Black (1980) by adding 4 drops of 50% silver nitrate and 2% gelatin on slides. The slides were then sealed with cover glasses and incubated at 60°C for 5 minutes. After that the slides were soaked in distilled water until the cover glasses were separated.

Chromosome checking

Twenty clearly observable cells with well spread chromosomes of each male and female were selected and photographed under Olympus Bx63 microscope. Metaphase figures were analyzed according to the chromosome classification of Turpin and Lejeune (1965). The length of the short arm chromosome (Ls) and the long arm chromosome (Ll) were measured and the length of the total arm chromosome (LT, LT = Ls + Ll) was calculated. The relative length (RL), the centromeric index (CI), and standard deviation (SD) of RL and CI were estimated. The CI (q/p + q) between 0.50-0.59, 0.60-0.69, 0.70-0.89, and 0.90-0.99 were described as metacentric, submetacentric, acrocentric and telocentric chromo-

somes, respectively. The fundamental number (NF, number of chromosome arms) was obtained by assigning a value of 2 to metacentric, submetacentric and acrocentric chromosomes and 1 to telocentric chromosome. All parameters were used in karyotyping and idiogram.

RESULTS AND DISCUSSION

This is the first report on cytogenetic characterization using conventional staining and Ag-NOR banding techniques for *T. somphongsi*. The results indicated diploid chromosome number (2*n*) were found 50 in all studies samples as show in Fig. 2. This result is coincident with *T. heteromorpha* reports by Khuda 1979, and similar to another species in *Rasbora* (Post 1965; Manna and Khuda-Bukhsh 1977; Khuda-Bukhsh et al. 1979; Donsakul and Magtoon 1995; Donsakul and Magtoon 2002; Seetapan and Moeikum 2004; Donsakul et al. 2005; Donsakul et al. 2009; Yeesaem et al. 2019; Aiumsumang et al. 2021; 2022). From the previous report, most of cyprinid species have 2*n*=50, chromosome consisting of both mono- and bi-arm chromosomes. There is no observation of strange size chromosomes related to sex, which is in accordance to the author of this genus (Khuda 1979). The types chromosomes of *T. somphongsi* were 10 metacentric, 20 submetacentric, 12 acrocentric and 8 telocentric chromosomes. The mean values calculated from twenty mitotic metaphases showed the centromeric index of chromosome complements ranging from 0.569±0.001 to 1.000±0.000 (Table 1).

To our results, NORs could be observed in one pair of chromosomes in both male and female of *T. somphongsi*. The result demonstrated that the chromosome marker shows in the chromosome pair 2, which is metacentric chromosome (Fig. 3). An important characteristic of Nucleolar Organizer Regions (NORs) in fish is related to that it has inter- and intra-species polymorphism. NORs characters can be a cytogenetic marker for cytotaxonomic studies and also have been used for studying of phylogenetic relationships among the Cyprinid fishes (Amemyia and Gold 1988; Galetti Jr 1998; Almeida-Toledo et al. 2000). The important karyotype feature of *T. somphongsi* is the symmetrical karyotype, which were found in four types of chromosomes (metacentric, submetacentric, acrocentric, and telocentric chromosomes). Figure 4 show the idiograms from conventional staining and Ag-NOR banding techniques. The karyotype formula could be deduced as: 2*n* (diploid) 50=10m+20sm+12a+8t. The study on fish chromosomes is the basic knowledge which can be applied for the several fields such as classification, evolution, heredity, systematic (Gold et al. 1990; Ueda et al. 2001; Barat et al. 2002; Barat and Sahoo 2007), breeding, rapid production of inbred lines and cytotaxonomy (Kirpichnikov 1981). Furthermore, cytogenetic studies on fish have also been used as biological indicator to determine the ecological toxicology (Klinkhardt, 1993) and cytogenetic techniques have been widely applied to improve farmed stocks in many aquaculture species in the World (Beardmore et al. 2001; Desprez et al. 2003).

Here, we have that the karyotype of *T. somphongsi* is 2*n*=50 and might represent a derived character, probably also shared by all members of the *Trigonostigma* clade. Besides, our study is the first cytogenetic karyotype data to describe in detail the karyotypic features of the Som-

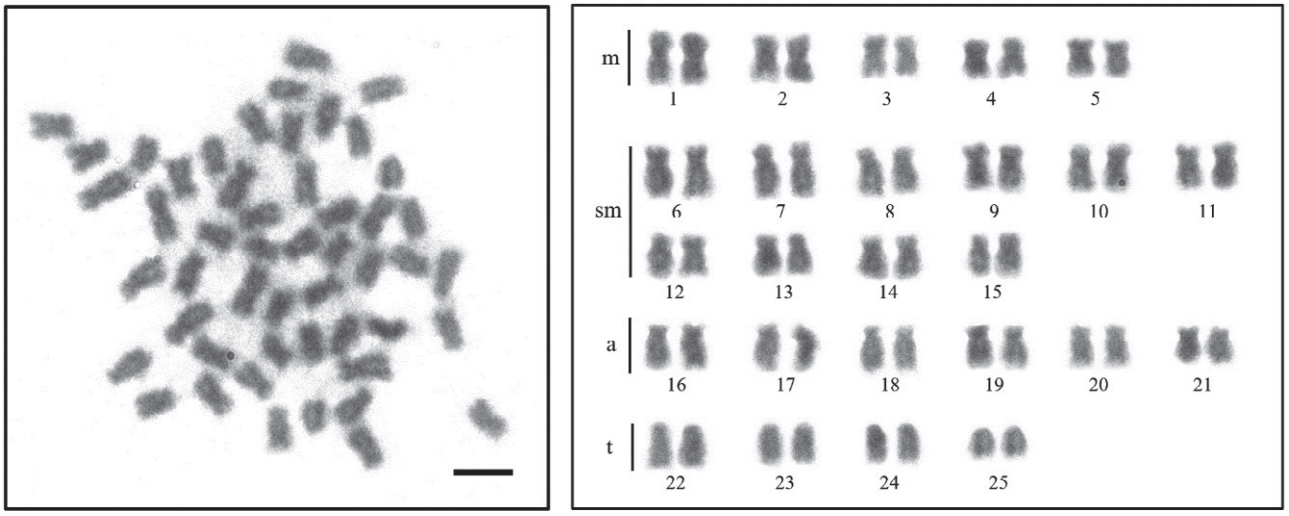

Figure 2. Metaphase chromosome plates and karyotypes of the *T. somphongsi* by conventional staining. Species share the karyotype composed of 50 chromosomes. Scale bar indicate 5 μm.

Table 1. Karyomorphological details of *T. somphongsi* from 20 metaphases chromosome, 2*n* (diploid)=50.

Chro. pair	Ls	Ll	LT	RL ± SD	CI ± SD	Chro. size	Chro. type
1	2.651	2.965	5.616	0.047±0.002	0.569±0.001	Large	metacentric
2	2.547	2.882	5.429	0.045±0.002	0.574±0.001	Large	metacentric
3	2.433	2.716	5.149	0.043±0.003	0.571±0.002	Large	metacentric
4	2.337	2.709	5.046	0.040±0.001	0.583±0.002	Medium	metacentric
5	2.927	2.609	5.536	0.039±0.001	0.583±0.002	Medium	metacentric
6	2.800	3.982	6.782	0.045±0.002	0.688±0.004	Large	submetacentric
7	2.692	3.237	5.929	0.044±0.001	0.680±0.001	Large	submetacentric
8	2.764	3.753	6.517	0.042±0.004	0.682±0.002	Large	submetacentric
9	2.985	3.706	6.691	0.040±0.004	0.674±0.002	Large	submetacentric
10	2.597	3.646	6.243	0.037±0.005	0.694±0.003	Medium	submetacentric
11	2.564	3.480	6.044	0.035±0.003	0.692±0.005	Medium	submetacentric
12	2.488	2.842	5.330	0.030±0.003	0.653±0.001	Medium	submetacentric
13	2.488	2.842	5.330	0.030±0.003	0.653±0.001	Medium	submetacentric
14	2.293	4.470	6.763	0.029±0.008	0.669±0.002	Medium	submetacentric
15	2.121	3.942	6.063	0.025±0.003	0.664±0.003	Medium	submetacentric
16	0.549	3.998	4.547	0.041±0.001	0.898±0.004	Large	acrocentric
17	0.404	3.565	3.969	0.038±0.001	0.894±0.004	Large	acrocentric
18	0.354	3.445	3.799	0.038±0.001	0.871±0.004	Large	acrocentric
19	0.344	3.014	3.358	0.037±0.001	0.874±0.004	Medium	acrocentric
20	0.175	2.985	3.160	0.035±0.001	0.872±0.004	Medium	acrocentric
21	0.168	2.014	2.182	0.031±0.001	0.890±0.004	Medium	acrocentric
22	0.000	4.624	4.624	0.029±0.001	1.000±0.000	Large	telocentric
23	0.000	3.615	3.615	0.027±0.001	1.000±0.000	Large	telocentric
24	0.000	3.550	3.550	0.026±0.001	1.000±0.000	Medium	telocentric
25	0.000	2.989	2.989	0.026±0.001	1.000±0.000	Medium	telocentric

Remarks: Ls=short arm chromosome, Ll=length of long arm chromosome, LT=length of total chromosomes, RL=relative length, CI=centromeric index, SD=standard deviation.

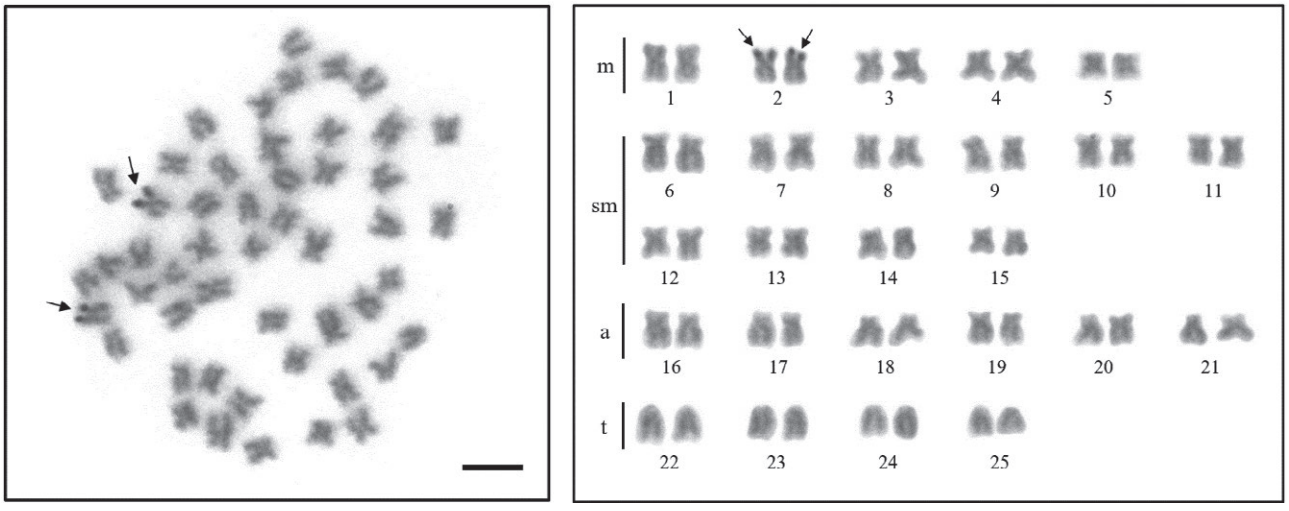

Figure 3. Metaphase chromosome plates and karyotypes of the *T. somphongsi*. The arrows indicate NOR banding by Ag-NOR staining technique. Scale bar indicate 5 μm.

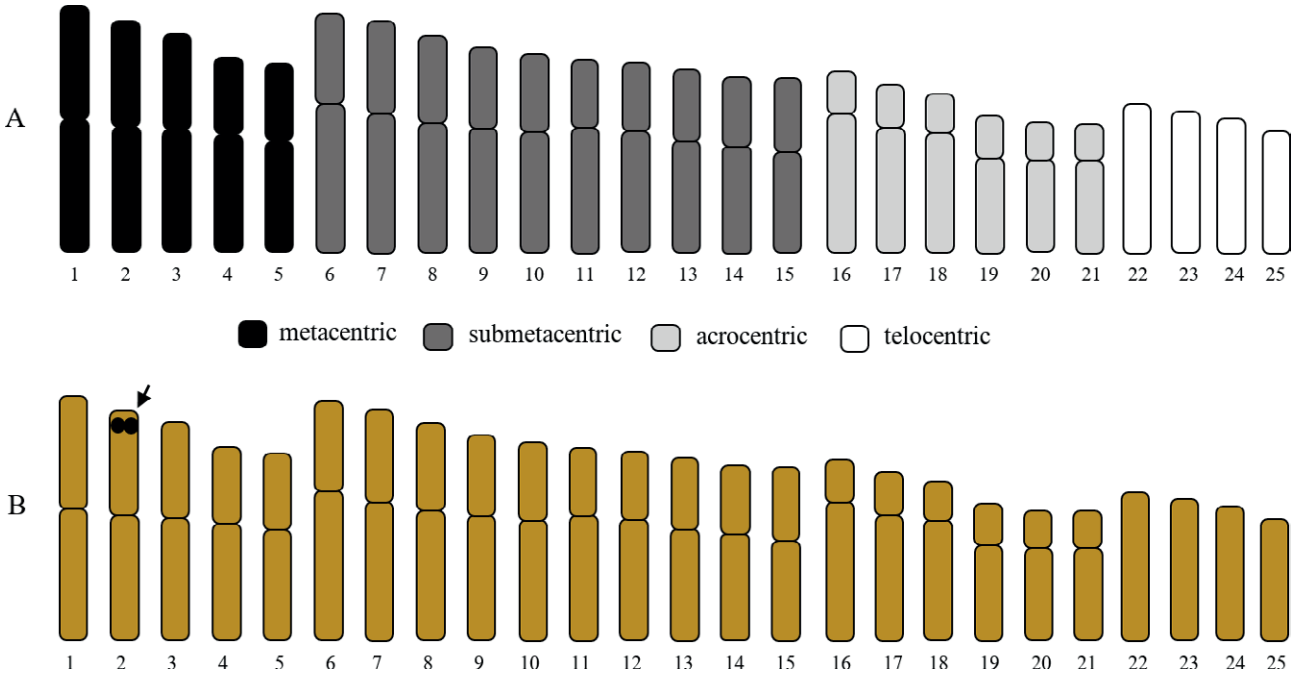

Figure 4. Idiogram showing lengths and shape of chromosomes of the *T. somphongsi*, *n* (haploid)=50, by conventional staining (A) and Ag-NOR staining technique (B). The arrows indicate NOR banding.

phong's rasbora species. The data can be a support for the investigation of chromosomal cytotaxonomy evolutionary history of relationships, conservation and on breeding practices within *Trigonostigma*.

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Evaluation of the evolutionary process within *Populus caspica* species from Hyrcanian forests by karyotype analysis

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Abstract. Caspian poplar (*Populus caspica* Bornm.) is distributed exclusively in the Hyrcanian forests. Hyrcanian forests are the final remnants of Tertiary temperate deciduous forests in Western Eurasia and worldwide. This species plays a significant ecological role in the protection of the natural environment in Hyrcanian forests. In this research, chromosome number and karyotype details of 11 populations of the species were investigated for the first time, using fresh root cuttings collected from mature trees in different parts of the forest, located in the northern parts of Iran. Pretreatment, fixation, hydrolyzing, and staining were conducted by α-bromonaphthalene, carnoy's solution, 1N HCl, and hematoxylin agent, respectively. Chromosomal data were analyzed according to a nested model based on a completely randomized design. Chromosome numbers of all of the populations were the same as $2n = 38$, which mostly were medium region and sub-metacentric types. Significant differences were observed between the provinces and populations, based on chromosome length grand means, arm ratios and centromere indices. The results demonstrated that structural rearrangement has occurred within the studied populations and indicated an active evolutionary process within and between populations of the species due to natural hybridization. Also, these results showed that artificial inter-specific hybridization between the *P. caspica* and its relative species can be employed to broaden the ecological zone of the species.

Keywords: asymmetry indices, chromosome number, karyotype, *Populus caspica.*

INTRODUCTION

Hyrcanian forests inscribed on the UNESCO World Heritage List contain the final remains of tertiary wide-leaved forests in western Eurasia and worldwide (UNESCO World Heritage Centre 2019; Bayranvand et al. 2017; Alipour et al. 2023). Hyrcanian forests are located between the north of the Alborz Mountain chains and the Caspian sea (Iran and Caucasus) (Sabeti, 1994). In Iran, which accounts for most of the hotspots of Iranian-Anatolian biodiversity, 30

percent of vascular plant species are endemic (Noroozi et al. 2018). In flora of Iran only two species, *Populus caspica* Bornm., and *P. euphratica*, are native and the others (*P. alba*, *P. deltoides*, *P. nigra* and hybrid species (*P. canadensis*)) are widely cultivated at different parts of Iran (Maassoumi et al. 2011). Caspian poplar (*P. caspica*), a member of the Salicaceae family, is classified as endangered in Iran (Jalili and Jamzad, 1999; Alipour et al. 2021). Many species including *P. caspica* have survived periods of glaciation in the Hyrcanian forests (Qin et al. 2017; Mohammadi et al. 2019). Poplars are so noteworthy in agroforestry industry because of their fast growth, desirable figure, as well as in providing wood, fiber, fuel-wood, and other forest products (Stettler, 2009; Evans, 2014). Natural habitats of *P. caspica* have largely been destroyed through environmental conditions and human activities like planting non-indigenous species, and agricultural usage (Khoshravesh et al. 2009). Habitat degradation has impacted the restoration of *P. capsica* due to unsuitable seedbed conditions (Asadi and Mirzaie-Nodoushan, 2011).

Comparative genomics and sequencing have been done in several poplar species that demonstrated high genetic diversity and frequent interspecific hybridization among the species (Li et al. 2023). Other karyological studies showed that in *Populus* genus, with a basic haploid chromosome number of 19, diploidy is predominant. Due to the small chromosomes, karyotype information has been reported for several *Populus* species (Islam-Faridi et al. 2009; Liu et al. 2021). Hence, in recent years fluorescence *in situ* hybridization (FISH) has been employed for identifying chromosomes in poplars to compare the karyotype and similarity of chromosome structure among different species (Xin et al. 2020; Kim et al. 2020). Apart from *P. euphratica*, there is no information on the number of chromosomes and karyotypic indices of the *Populus* species, including *P. caspica,* in Iran. Therefore, this study was undertaken to provide cytological information on the species based on conventional methods that can be useful as a guide in future breeding programs and evaluation of evolutionary process.

MATERIALS AND METHODS

Plant materials were obtained from trees in 11 different parts of two provinces, Gilan and Mazandaran, located in north part of Iran. Root tip meristems, collected from cutting grown under hydroponic conditions, were pre-treated with 0.5% α-bromonaphthalene for 1 hour in refrigerator, then fixed in a mixture of ethanol alcohol and glacial acetic acid (3:1 v/v) for 16 hours. The fixed samples were washed 2–3 times and preserved in 70% ethanol. Root tips were hydrolyzed with 1N HCl solution at 60°C for 6 minutes, stained in hematoxylin reagent for 2 hours at 60°C, and finally squashed in 45% acetic acid (v/v) (Mirzaie-Nodoushan and Asadi-Corom, 2002). Somatic chromosomes were photographed using digital camera and the chromosomes were measured via Ideokar 1.2 software (Ghader Mirzaghaderi and Karim Marzangi, 2015). Based on the centromere position nomenclature of chromosomes was described (Levan et al. 1964). Along with chromosomal dimensions several chromosomal parameters, such as Arm ratio, r-value, Relative length of chromosome, Form percentage of chromosome, centromeric index (CI=S/TL) were calculated. As well as, asymmetry indices were calculated using Intra-chromosomal asymmetry index (A1) (Zarco, 1986), Inter-chromosomal asymmetry index (A2) (Zarco, 1986), Symmetry index (S%) (Watanabe et al. 1999), Total form percentage (TF%) (Huziwara, 1962), difference of range relative length (DRL) and Stebbins class asymmetry index (Stebbins, 1971).

Chromosomal data were analyzed using a nested model based on a completely randomized design , regarding the provinces, populations and chromosomes as the three nested factors with three replications of well-spread metaphasic plates. In this case provinces are considered as factor A, populations as factor B, nested within factor A, which is shown in statistical point of view as, populations (A), and chromosomes as factor C, nested within factor B, (chromosomes (B A)). Duncan multiple range test was carried out for classifying the populations by SAS 9.4 software. Cluster analysis was performed in order to classify the populations based on chromosomal measures and karyotypic indices using Ward method, by JMP 13.2.0 software.

RESULTS

The chromosome counting revealed that all of the studied populations were diploid, containing a total of 38 chromosomes ($2n$ = 38) with a single pair carrying the satellites, located on the short arms of the chromosomes (Fig. 1). Chromosome length grand mean and arm ratio (AR) showed a significant difference ($p \leq 0.05$) between the provinces, while arm ratios and centromere indices were different ($p \leq 0.01$) between the populations (Table 1). Size of the chromosomes among the studied populations varied from 0.65 μm (Tash12) to 2.90 μm (Tash2) and from 0.67 μm to 2.32 μm in Mazandaran and Gilan populations, respectively (Table 2).

The karyotype formula of the studied populations is presented in Table 1. In both provinces, medium region

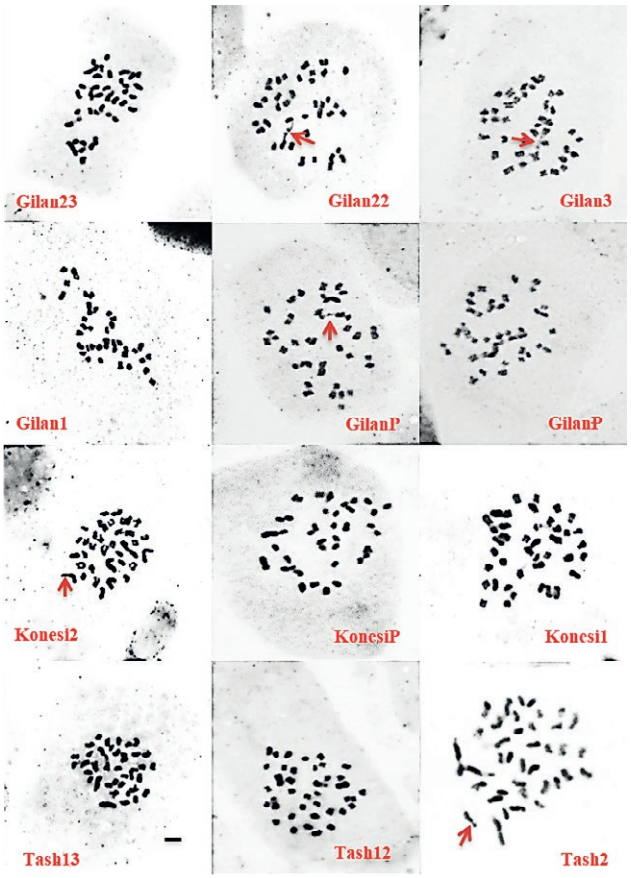

Figure 1. Mitotic metaphase chromosomes of the studied populations of *Populus caspica* in Iran (Arrows are pointing to satellites; Bar=100 μm).

(m) type chromosomes were the dominant type, especially in Gil22 and Tash13. That's why their karyotypes are symmetrical. All populations possessed one to seven chromosomes of sub-metacentric (sm) type. Sub-terminal region (st) type was observed only in two populations of Mazandaran. The most asymmetrical karyotype was found in the Konesi2 population of the same province (21m+14sm+3st). In this population, medium point and terminal point types of chromosomes were also found in several single plants (23m+4sm+9st+2T+sat; 1M+34m+1sm+2st) (Table 3). Also based on chromosome characteristics and karyotypic indices, the plant populations were clustered in three groups. The konesi2 population, with the most asymmetrical karyotype, was clustered into a single group. (Fig. 2).

DISCUSSION

In most modern poplars, the cell nucleus typically contains two sets of 19 ($2n$ = 38) chromosomes (Chen et al. 2005; Shou-Gong et al. 2005) that agree with the results obtained by this research. Triploids with three sets of chromosomes ($2n$ = 57) (Peto, 1983) and tetraploids ($2n$ = 76) have also been identified in section *Populus* (Einspahr et al. 1964; Every and Wiens 1971). According to IPCN and literature surveys, triploidy in *P. nigra* and *P. canadensis* (Shou-Gong et al. 2005; Chen et al. 2005) and aneuploidy in two varieties of *P. alba* have been reported (IPCN, http://www.tropicos.org/Project/IPCN). The presence of only one couple carrying one pair of satellite, represents the basic profile of this species same as other species of poplars.

Chromosome numbers and chromosome rearrangements are the major source of karyotype evolution and closely related species maintain a similar chromosome number. Despite the similar chromosome number in the studied populations, a structural diversification was observed in the studied populations. Difference in the karyotypic formula within the species indicates that chromosome structural changes have occurred. Pericentric inversions is one of the most common mechanisms related to karyotypic variation (Molina and de Freitas Bacurau, 2006; Carbone et al. 2014). They can shift the position of the centromere within a chromosome and cause the arm ratio to change. Inverted chromosomes

Table 1. Mean squares resulted from nested model analysis of variance of chromosome parameters of *Populus caspica* populations.

Source of variation	DF	L (μm)	S (μm)	TL^{M} (μm)	AR	r-Value	RL%	F%	CI
A: provinces	1	0.37*	0.004^{ns}	0.0.07*	0.97*	0.002^{ns}	0.00^{ns}	0.006^{ns}	0.001^{ns}
B: populations (A)	9	0.16^{ns}	0.11^{ns}	0.48^{ns}	1.31**	0.06**	1.68^{ns}	0.06^{ns}	0.009**
C: Chromosomes (B A)	198	0.13**	0.07**	0.39**	0.14^{ns}	0.01^{ns}	0.1**	0.4**	0.002^{ns}
Error	418	0.007	0.004	0.01	0.21	0.02	0.02	0.02	0.002
CV%		13.65	14.46	10.16	31.98	17.04	5.84	12.52	11.27

L= length of the longest chromosome, S= length of the shortest chromosome, TLM = grand mean of chromosome length, AR= L/S, r-Value= S/L, RL%= relative length of chromosome, F%= form percentage of chromosome, CI= centromeric index.
**: significant difference at 1% level, *: significant difference at 1% level, ns: no significant difference.

Table 2- Means of mitotic features of the studied populations of *Populus caspica.*

Provinces	Populations	L (μm)	S (μm)	TL^M (μm)	AR	r-Value	RL%	F%	CI
Gilan	Gil13	0.59^{d}	0.43^{d}	1.02^{g}	1.43^{b}	$0.74b^{cd}$	2.63^{a}	1.11^{abc}	0.42^{ab}
Gilan	Gil23	$0.66b^{c}$	0.47^{c}	1.13^{d}	1.47^{b}	$0.74b^{cd}$	2.63^{a}	1.09^{bc}	0.42^{b}
Gilan	Gil22	0.67^{bc}	0.51^{b}	1.18^{bc}	1.34^{b}	0.78^{abc}	2.63^{a}	1.14^{ab}	0.43^{ab}
Gilan	Gil31	$0.66b^{c}$	0.52^{b}	1.18^{c}	1.31^{b}	0.80^{a}	2.63^{a}	1.16^{a}	0.44^{a}
Gilan	GilP	0.64^{c}	0.46^{c}	1.10^{de}	1.47^{b}	0.73^{cd}	2.63^{a}	1.09^{bc}	0.41^{b}
Mazandaran	Konesi1	0.61^{d}	0.44^{cd}	1.05^{fg}	1.46^{b}	0.74^{abcd}	2.63^{a}	1.11^{abc}	0.42^{ab}
Mazandaran	Konesi2	0.69^{b}	0.46^{c}	1.15^{cd}	1.87^{a}	0.70^{d}	2.63^{a}	1.05^{c}	0.40^{c}
Mazandaran	KonesiP	0.61^{d}	0.46^{c}	1.07^{ef}	1.40^{b}	0.77^{abc}	2.63^{a}	1.14^{ab}	0.43^{ab}
Mazandaran	Tash12	0.60^{d}	0.45^{cd}	1.05^{fg}	1.39^{b}	0.77^{abc}	2.63^{a}	1.13^{ab}	0.43^{ab}
Mazandaran	Tash13	0.69^{b}	0.53^{ab}	1.23^{b}	1.35^{b}	0.79^{ab}	2.63^{a}	1.15^{ab}	0.44^{ab}
Mazandaran	Tash23	0.76^{a}	0.55^{a}	1.31^{a}	1.42^{b}	0.75^{abc}	2.63^{a}	1.11^{abc}	0.42^{ab}

L= length of the longest chromosome, S= length of the shortest chromosome, TL^M =grand mean of chromosome length, AR= L/S, r-Value= S/L, RL%= relative length of chromosome, F%= form percentage of chromosome, CI= centromeric index, Similar letters within each column, indicate no significant difference between the populations at 5% level.

Table 3. Karyotypic parameters of the studied populations of *Populus caspica.*

Provinces	Populations	Stebbins	FK	A1	A2	S%	TF%	DRL%
Gilan	Gil1	1B	34m+4sm	0.26	0.09	33.77	42.05	3.34
Gilan	Gil23	2B	32m+6sm	0.26	0.05	32.09	41.55	3.59
Gilan	Gil22	1B	37m+1sm (**2sat**)	0.22	0.08	34.33	43.49	3.40
Gilan	Gil3	1B	32m+6sm (**2sat**)	0.20	0.06	32.68	43.96	3.48
Gilan	GilP	1B	34m+4sm (**2sat**)	0.27	0.06	30.68	41.58	3.78
Mazandaran	Konesi1	1B	32m+6sm	0.26	0.09	33.42	42.01	3.44
Mazandaran	Konesi2	2B	21m+14sm+3st (**2sat**)	0.30	0.13	28.28	39.93	4.08
Mazandaran	KonesiP	2B	34m+3sm+1st	0.23	0.11	32.53	43.21	3.26
Mazandaran	Tash12	1B	35m+3sm	0.23	0.11	34.26	42.88	3.25
Mazandaran	Tash13	1B	36m+2sm	0.21	0.08	32.31	43.59	3.54
Mazandaran	Tash2	1B	31m+7sm (**2sat**)	0.25	0.05	26.04	42.00	4.31

KF= karyotypic formulae, A1= Intra-chromosomal asymmetry index, A2= Inter-chromosomal asymmetry index, S%= Symmetry index, TF%= Total form percentage, DRL%= Differences between the maximum and minimum relative length of the chromosomes; sat= satellite.

have the potential to contribute to asymmetrical bivalents (Singh, 2017). In fact, chromosomal rearrangements are often the main source of karyotypical evolution and would indicate an active evolutionary process within and between populations of the species (Mirzaie-Nodoushan et al. 2006; Xin et al. 2020). In Hyrcanian forest, the natural hybridization and large-scale interspecific hybridization between *P. caspica* and other cultivated poplar species, such as European *P. alba*, *P. nigra*, *P. deltoids* (North American poplar), is documented using cpDNA (chloroplast DNA) and ITS (Internal Transcribed Spacer) fragments (Yousefzadeh et al. 2019). Inter-chromosomal translocation is another factor for chromosomal rearrangements and diversity of karyotypic parameters in *P. caspica* as a result of cross-pollination (Fig. 2) while this result is contrary to previous study by Xin et al. (2020). By chromosome painting probes they demonstrated that no chromosomal rearrangements on any of the 19 chromosomes among some species of *Populus* including *P. euphratica* and *P. deltoids* have occurred. On the other hand, the potential for intact or largely partial chromosome transfer between poplar hybrids has been proposed by Liu et al. (2021) using labeled telomeres, rDNA, and repetitive sequences as probes, that supports the findings in the present study.

The existing variation within the species based on chromosomal parameters was remarkable. As it was mentioned earlier, sexual and clonal reproduction of *P. caspica* is limited and the species is endangered in the area of its habitation in the country. This impor-

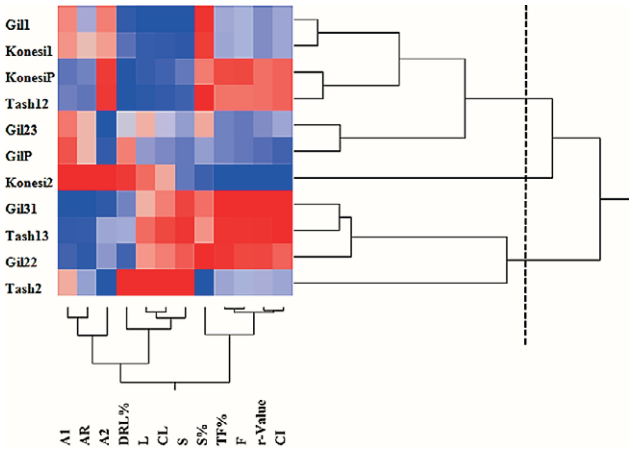

Figure 2. Diversity between the populations of *Populus caspica* based on chromosome characteristics and karyotypic indices (Ward method).

tant point should be regarded as a major restriction of the species. Along with natural hybridization, artificial inter-specific hybridization between the *Populus* species was suggested by other researchers (Mirzaie-Nodoushan et al. 2015) for inducing genetic variation and broadening the genetic basis of poplar germplasm. This suggestion can be employed on *P. caspica* and its relative species, with the same chromosome number, to broaden its genetic basis, as well as broadening the ecological zone of the species which is restricted to the northern part of Iran and Caucasus.

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Competing Interests: The Author(s) declare(s) no conflict of interest.

Chromosomal and genome size variations in Opium poppy (*Papaver somniferum* L.) from Afghanistan

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Abstract. The genus *Papaver* classified in the Papaveraceae family, is a valuable, non-alternative medicinal plant which has illustrated a massive variety of pharmacologically important alkaloids. Chromosomal and monoploid genome size diversity of seven populations collected from different districts of Balkh Province in northern parts of Afghanistan were studied. All populations were diploid, six of which (P1-P6) had 22 chromosomes, while P7 had 20 larger chromosomes. The mean chromosome length (CL) of P1-P6 populations was 1.32 µm (0.91-1.74 µm), but that of P7 population was 2.24 µm. The results of flow cytometric analysis showed that the mean monoploid 2Cx DNA of P1-P6 populations was 5.701 pg (5.574-5.901 pg), whereas that of P7 population was 5.795 pg, confirming intraspecific variation. This study is being reported for the first time from the northern part of Afghanistan's opium cultivation area, and P7 population is also being reported for the first time in terms of chromosome number. Valuable information on Cytogenetics can be used in some research fields, including polygenetic analysis, taxonomic relationships, evolutionary characteristics, and plant breeding.

Keywords: *Papaver somniferum*, chromosome, monoploid genome size, 2Cx DNA, flow cytometry, Balkh province, Afghanistan.

1. INTRODUCTION

Opium poppy (*Papaver somniferum* L., $2n = 2x = 22$) is one of the oldest cultivated medicinal plants that has been used for thousands of years (Askitopoulou *et al*., 2002; Vu *et al*., 2022). Its origin and domestication is not clear, but archeological findings and references prove that the Mediterranean is the origin of the poppy plant from the middle of the 6th millennium BC (Askitopoulou *et al*., 2002; Salavert *et al*., 2018; Vu *et al*., 2021; Jesus *et al*., 2021). Recently, poppy has been cultivated, both as a licit and illicit crop, in Asia, Europe, Oceania and South America as a main source of benzylisoquinoline alkaloids (BIAs) (Askitopoulou *et al*., 2002; Beaudoin and Facchini, 2014; Guo *et al*., 2018; Vu *et al*., 2021). The word poppy has been used for many species of the Papaveraceae family, while opium word has been used for the air-dried latex extraction obtained from *Papaver somniferum* L. cap-

sules, one of the most useful plant species belonging to this family (Labanca *et al*., 2018). Opium poppy is one of the non-alternative sources of morphine, codeine, noscapine (Khan *et al*., 2011), and semisynthetic derivatives, including oxycodone and naltrexone (Carlin *et al*., 2020; Pei *et al*., 2021). Morphinan-based sedatives are obtained from opium poppy (Guo *et al*., 2018). There are about 600 species in the Papaveraceae. The majority of those are cultivated in gardens and their karyotypes are studied. In comparison, the meiotic chromosome numbers differ from 7 to 11(Sugiura, 1939). According to the latter report, the first karyological studies of Papaveraceae were done by Nemec (1910) on *Corydalis pumila* and next Tahara on *Papaver rhoeas, oriental*, and *somniferum*. Afterwards, Yasui (1921) and Ljungdahl (1922) reported that *Papaver* species are the chief studies in the Papaveraceae family. From which, meiotic karyotype studies of *Papaver somniferum* revealed that all populations were diploid with 11 chromosomes (Kaul *et al*., 1979; Rezaei *et al*., 2014). Estimation of *Papaver somniferum* genome size by flow cytometry is an easy and rapid technique that allows accurate value of nuclear DNA content (Kyrylenko *et al*., 2005). Some studies were carried out on the Papaveraceae family for genome size estimation, e.g. it was reported that the genome size of *P. somniferum* was 6.46 pg (Kyrylenko *et al*., 2005) and this amount for *P. bracteatum* was 6.15 pg (Tarkesh Esfahani *et al*., 2016). Hence, the key objective of the current study was to investigate the chromosomal and genome size variations in seven populations of opium poppy (*Papaver somniferum* L.) medicinal plant.

2. MATERIALS AND METHODS

2.1. Plant materials

The ripped seeded capsules of seven populations of *Papaver somniferum* were collected from seven different districts of Balkh Province, Afghanistan in 2020. Seeds were provided from mature capsules and the seeds of each individual plant were collected separately and kept in small plastic bags. The characteristics of the local information of the collected sites and geographic distribution are presented in Table 1 and Figure 1.

Table 1. Collected sites locations of *Papaver somniferum* populations of Northern part of Afghanistan in this study.

Population codes	Locality	Latitude (N)	Longitude (E)	Altitude (m)
P1	Chahar Kint, Balkh	36°20'33.43"	67°31'23.38"	1822
P2	Balkh, Balkh	36°45'54.62"	66°53'19.24"	341
P3	Kishindeh, Balkh	35°48'11.83"	67°05'38.92"	1862
P4	Chimtal, Balkh	36°28'27.42"	66°57'3.47"	553
P5	Khulm, Balkh	36°42'50.09"	66°57'7.92"	365
P6	Sholgara, Balkh	36°22'12.24"	66°53'29.67"	551
P7	Chahar Bolak, Balkh	36°48'5.64"	66°57'3.68"	335

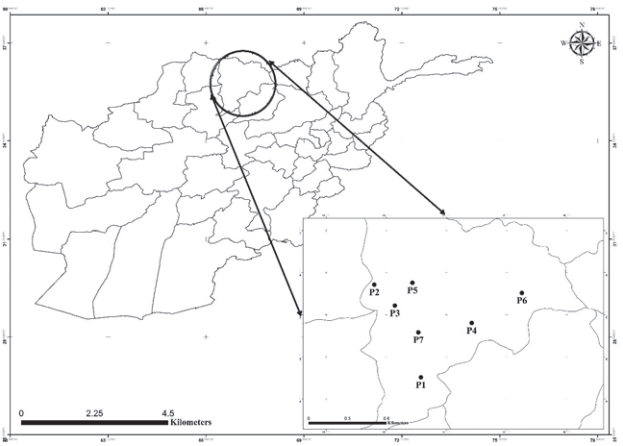

Figure 1. Geographic distribution of sampled *Papaver somniferum* on the map of Afghanistan using ArcGIS.

2.2. Cytological preparation

Seeds of seven *Papaver somniferum* L. populations were germinated on thick layers of petri paper in a glass petri dish at 23 ± 2 °C (Kaul *et al*., 1979; Tarkesh Esfahani *et al*., 2020). Seeds were sterilized by immersing in 70% (v/v) ethanol three times and 30s each time, followed by sodium hypochlorite 5% (v/v) for 6 min, and rinsed by distilled water for 3 times. The sterilized seeds were then transferred to two layers of moisturized filter paper in glass petri dishes and irrigated regularly by distilled water until germination (Tarkesh Esfahani *et al*., 2016). The seeds started germinating after 72 h in 16 h light and 8 h dark conditions at 23 ± 2 °C. Since pretreatment is necessary (Rezaei *et al*., 2014), so the 2 cm-long roots were first cold pretreated for 1.5 h at 4 °C, followed by chemical pretreatment in 0.05 M aqueous colchicine solution for 2.5 h (Ahmadi-Roshan *et al*., 2016). They were then fixed in acetic acid: alcohol (1:3) for 1 h. The roots were hydrolyzed for 15 min in 1M HCl, followed by staining with aceto-orcein 2% (w/v) for 2 h (Chowa *et al*., 2020; Sayadi *et al*., 2021; Najafi *et al*., 2022). Squash method was applied at 45% HCl (v/v) for preparing slides. Photomicrographs were captured by a DP12 digital camera (Olympus Optical Corporation, Tokyo, Japan) appointed to a BX50 Olympus microscope (Olympus Optical Corporation, Ltd., Tokyo, Japan).

Figure 2. Growing stage of seven population of *Papaver somniferum* in plate culture after 70 d (a). Transfer of every single population in separate pots in age 95 d (b). Growing stage of *Solanum lycopersicum* cv. Stupicke (2C = 1.96 pg DNA) the reference standard plant (c).

The chromosome length (CL) was measured using MicroMeasure software version 3.3.

To estimate the genome size, the seeds were cultured in a plate culture, containing sterile perlite and coco-peat under room temperature conditions. After two months, the grown plants having four developed leaves were transferred to separate pots. One cm^2 of young and well developed leaves of both *Papaver somniferum* plants and *Solanum lycopersicum* cv. Stupicke; 2C = 1.96 pg DNA (Doležel *et al*., 1998) as an internal reference standard plant were chopped simultaneously by a sharp razor blade in a glass petri dish, containing one ml of Woody Plant Buffer (WPB) (Loureiro *et al*., 2007; Tarkesh Esfahani *et al*., 2020; Sayadi *et al*., 2022). The resultant nuclear suspension was filtered through a green Partec 30 µm-nylon mesh (Partec, Munster, Germany), followed by treating with 50 µg ml^{-1} RNase (Sigma-Aldrich Corporation, MO, USA) and 50 µg ml^{-1} Propidium Iodide (PI, Fluka) as DNA staining agent, and then incubated for 2 min at room temperature. To determine the nuclear monoploid 2Cx DNA, the nuclei suspension was analyzed by a BD FACSCanto II flow cytometer (BD Biosciences, Bedford, MA, USA), using BD FACSDivaTM Software. Output data were then transferred to FloMax Software for Partec Flow Cytometer 2.4.1. The measurements of relative fluorescence intensity of stained nuclei were performed on a linear scale, analyzing at least 5,000 nuclei for each sample. The absolute DNA amount of a sample was calculated based on the values of the G1 peak means (Doležel *et al*., 1998; Bennett *et al*., 2000; Brown and Wittwer, 2000; Loureiro *et al*., 2007; Abedi *et al*., 2015; Tarkesh Esfahani *et al*., 2020; Abbasi-Karin *et al*., 2022; Sayadi *et al*., 2022) as follows:

Sample 2Cx DNA (pg) = (Sample G1 peak mean/Standard G1 peak mean) × Standard 2C DNA (pg)

Table 2. ANOVA of chromosome length (CL) and monoploid genome size (2Cx DNA; pg) of *Papaver somniferum* Populations.

S.O.V.	Df	MS CL	Df	MS 2C DNA (pg)
Population	6	36.215**	6	0.04174ns
Error	373	0.426	14	0.02597
Total	379		20	
CV%		21.7		8.93

ns Non significant difference ($P > 0.05$); ** significant difference ($P < 0.01$)

2.3. Statistical analyses

The normality test was first applied to chromosome length (CL) and genome size data, followed by *ANOVA*, using a completely randomized design (CRD) with five and three replications, respectively. The least significant difference (LSD) mean comparisons were carried out, using the general linear model (GLM) procedure in SAS 9.1 software (SAS Institute Inc 2009).

3. RESULTS

Karyotypic study results show that all of the examined seven opium poppy (*Papaver somniferum* L.) populations of Balkh Province, Afghanistan were diploids; six among which possess $2n = 2x = 22$ chromosomes, while the other one had $2n = 2x = 20$ chromosome (Figure 3, Table 3). This study has being reported for the first time

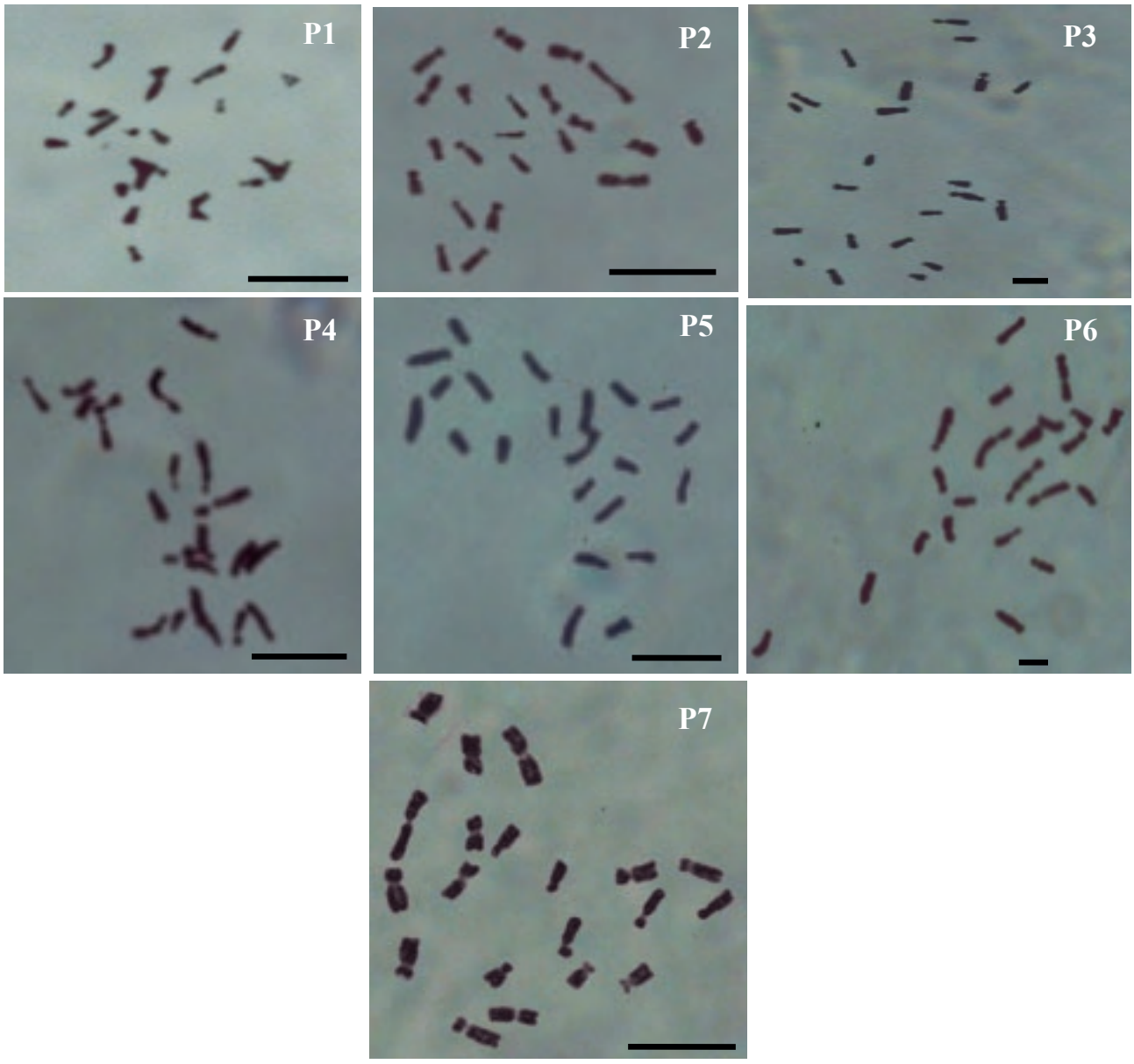

Figure 3. Karyotypes of somatic chromosomes of *Papaver somniferum* populations. Scale bars = 5 µm.

on opium cultivation area in Balkh Province, the northern part of Afghanistan. *ANOVA* results indicate significant differences ($P < 0.01$) for CL between the studied populations, showing intraspecific diversity (Table 2). The mean CL of all populations are shown in (Table 3). The mean CL of the first six populations (P1-P6) with $2n = 2x = 22$ chromosomes was 1.32 µm, ranging from 0.91 µm (P1) to 1.74 µm (P6), but that of the P7 population was 2.24 µm (Table 3). The monoploid nuclear DNA contents of seven studied populations are shown in figure 4. The mean 2Cx DNA amount of the P1-P6 populations with 22 chromosomes was 5.701 pg, ranging from 5.574 pg (P5) to 5.901 pg (P1), while that of P7 population was 5.795 pg (Table 3). The coefficients of variation for G_0/G_1 peaks of all species were less than 5%. The *ANOVA* of genome size indicates non-significant differences ($P > 0.05$). Hence, to make sure of any possible difference, furthermore, LSD mean comparisons were carried out at 0.05 probability level, indicating significant differences between P1 and P3 and P5 (Table 3). The results of histogram analysis are complementary and confirm the karyotypic studies, indicating the diploid nature of the examined populations.

Table 3. Means (± SE) comparisons of chromosome length (CL) and monoploid genome size (2Cx DNA; pg) of *Papaver somniferum* populations from Balkh-Afghanistan

Population	Locality	2*n*	CL	2Cx DNA (pg) Mean ± Se	1Cx genome size (pg)	1Cx genome size (Mbp)
P1	Chahar Kint	22	$0.91^{f} \pm 0.030$	$5.901^{a} \pm 0.11$	2.95	2885.10
P2	Balkh	22	$1.50^{c} \pm 0.047$	$5.779^{ab} \pm 0.04$	2.89	2826.42
P3	Kishindeh	22	$1.08^{e} \pm 0.039$	$5.613^{b} \pm 0.03$	2.81	2748.18
P4	Chimtal	22	$1.41^{cd} \pm 0.045$	$5.716^{ab} \pm 0.08$	2.86	2797.08
P5	Khulm	22	$1.27^{d} \pm 0.033$	$5.574^{b} \pm 0.18$	2.79	2728.62
P6	Sholgara	22	$1.74^{b} \pm 0.062$	$5.625^{ab} \pm 0.08$	2.81	2748.18
P7	Chahar Bolak	20	$2.24^{a} \pm 0.074$	$5.795^{ab} \pm 0.06$	2.89	2826.42
Means		P1-P6	1.32	5.701	--	--
		P7	2.24	5.795		
$LSD_{1\%}$			0.32	0.28	--	--

Means followed by the same letter within (CL) and "2Cx DNA (pg)" columns indicate they are not significantly different at ($P > 0.01$) and ($P > 0.05$), respectively, using LSD test.

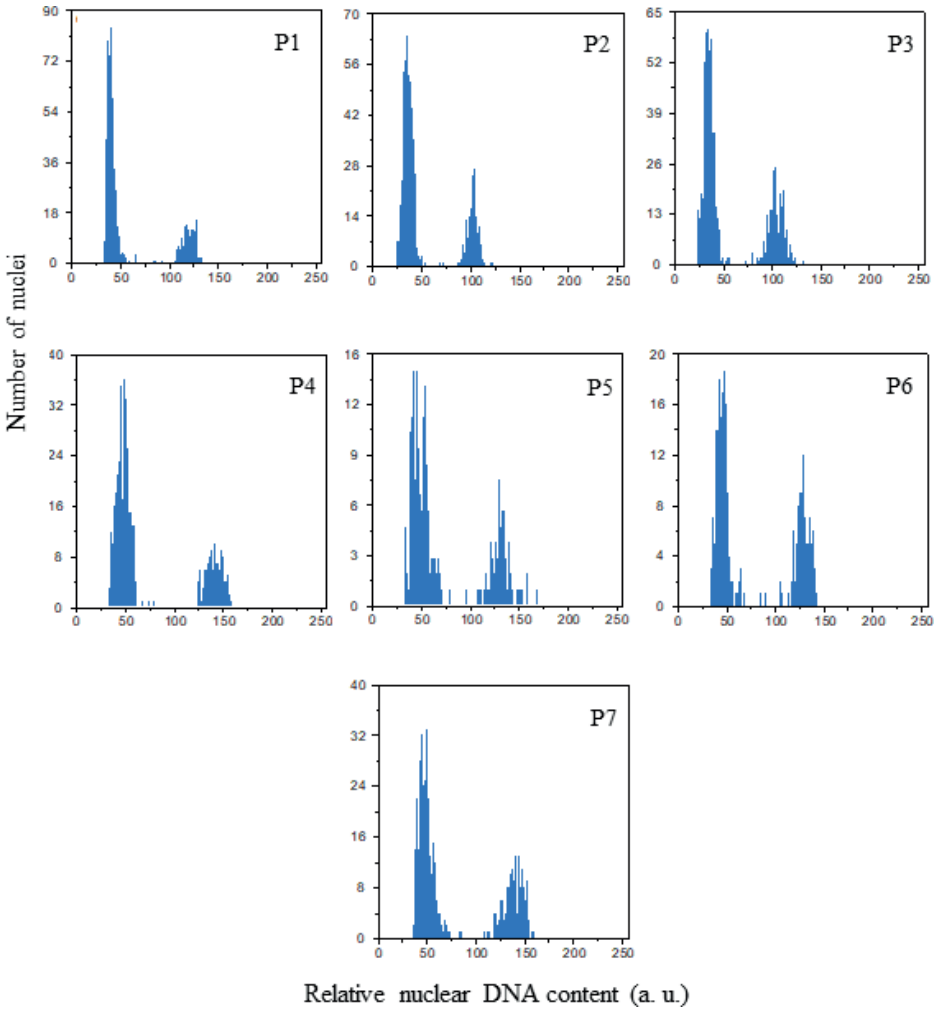

Figure 4. Flow cytometric histograms of 2Cx DNA content of seven *Papaver somniferum* populations. The left peaks refer to G_1 of the *Solanum lycopersicum* cv. Stupicke; 2C = 1.96 pg DNA internal reference standard and the right peak is G1 of the sample (*Papaver somniferum* L.).

4. DISCUSSION

Many pharmaceutical components and medical benefits have been reported for Opium poppy (Khan *et al*., 2011; Heydari *et al*., 2013; Labanca *et al*., 2018). Opium poppy is an important drug plant used in the manufacture of benzylisoquinoline and phenanthrene groups of alkaloids (Gümüşçü *et al*., 2008). For using the potential applicability, this plant still requires more research on its genetic characteristics as well as developing breeding methods. In the current study, we studied seven populations of opium poppy (*Papaver somniferum* L.) in terms of the chromosomal and genome size variations. Somatic chromosome morphology of *Papaver somniferum* shows that their chromosomes are numbered from 1 to 11 in order of differentiation in chromosome length (Kaul *et al*., 1979). The karyotype of cultured poppy plant root tips showed 22 chromosomes in all well spread root tip and shoot tip cells with more variation in length and centromere positions (Wakhlu and Bajwa, 1987). The karyotypic study in the present research showed that all populations were diploid and, in terms of chromosome numbers, P1-P6 had 22 and P7 had 20 chromosomes. The 22-chromosome number is in agreement with the studies conducted by Kaul *et al*. (1979) and Wakhlu and Bajwa, (1987), but not for the 20-chromosome P7 population. Considerable variation in somatic chromosome numbers of many plants, especially in the root tips, has been reported (Winterfeld., 2020; Mehravi *et al*., 2022). The first karyological studies of the Papaveraceae were done by Tahara on *Papaver somniferum, P. orientale*, and *P. rhoeas* which reported $2n = 22$ for poppy (*Papaver somniferum*) species (Sugiura, 1940). That is in exact conformity with P1-P6 populations in the current study, but differed from those in P7. On the other hand, in recent studies, the chromosome number of Iranian poppy (*Papaver bracteatum* L.) in diploid and in induced tetraploids showed 14 and 28, respectively (Tarkesh Esfahani *et al*., 2020), showing massive difference with that in the present study. Based on the obtained results of the current study, the P1-P6 populations were diploid with the base chromosome number of $x = 11$, the same base chromosome number of 11 was reported by previous studies (Sugiura, 1940; Kaul *et al*., 1979; Wakhlu and Bajwa, 1987; Tetenyi, 1994; Rezaei *et al*., 2014). Flow cytometry describes the use of this technique for the estimation of genomic DNA amount in cell nuclei (Doležel and Bartoš, 2005). In a research, the average 2C DNA content of all Persian poppy plants (*Papaver bracteatum*) was estimated as 6.15 ± 0.03 pg (Tarkesh Esfahani *et al*., 2016, 2020), indicating differences with that in the present study on *P. somniferum* populations. It can be noted that the 2Cx DNA content of *P. somniferum* species, having less variation was previously reported by researchers (Kyrylenko *et al*., 2005; Rezaei *et al*., 2014; Tarkesh Esfahani *et al*., 2016; Vu *et al*., 2021; *Pei et al*., 2021). The genome size of the first six populations (P1-P6) with 22 chromosomes was 5.701 pg (5.574-5.901 pg), which is similar to the previous report of Kyrylenko *et al*. (2005) in poppy species (*P. somniferum*) in terms of chromosome numbers possess 6.46 pg genomes size, showing a difference of 0.76 pg (13% reduction than that in the present study). By division of genome size by the number of chromosomes pg/chr, which was previously done in Mahdavi and Karimzadeh (2010) study on *Thymus* species (Lamiaceae), was also conducted in the current research. Hence, the genome size of the P1-P6 populations on the chromosome was equal to 0.259 pg/chr. Such statistics was calculated in the previous report by Kyrylenko *et al*. (2005) for *P. somniferum* with the same chromosome number to be 0.294 pg/chr, revealing 13% more than that in our six populations in the current study. More interestingly, 0.439 pg/chr was calculated in the Iranian poppy species (*P. bracteatum*) in the study of (Tarkesh Esfahani *et al*., 2020) which showed about 1.69 and 1.52 times increases compared to that in the first six populations and in the 7th population in the present study, respectively. The current study provides brand new information about genome size content diversity and karyotype in *P. somniferum* populations for the first time from the northern parts of Afghanistan that will help next researchers to consider whether other populations exist in other parts of this country.

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Karyological analyses in several Algerian populations of six species of the genus *Vicia* L. (*Fabaceae*)

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Abstract. As part of the evaluation and valorization of plant genetic resources of fodder and pastoral interest in Algeria, seventeen (17) natural populations belonging to six (06) species of the genus *Vicia* (*Vicia sativa*, *Vicia disperma*, *Vicia monardii*, *Vicia ohchroleuca*, *Vicia onobrychioides* and *Vicia lutea*), originated from different ecological regions in the North-Eastern of the country, were considered. The populations have been the subject of mitotic and meiotic studies. Haploid and diploid numbers and chromosome measurements were determined. Original results were observed for the first time in some species. In fact, chromosome counts have revealed some new chromosome numbers. The first number (2n=14, n=7) was observed in the endemic subspecies of Algeria, *V. ochroleuca subsp. atlantica* and in the species *Vicia onobrychioides*. The second number (n=6) was observed in the species *Vicia disperma*. Within *Vicia monardii*, the three new chromosome numbers, previously observed only in mitosis by our research team, were confirmed for the first time through the present meiosis study (n=6, 7 and 8), indicating that they are A type chromosomes. The base number x=7 is the most frequently observed number in the six *Vicia* species. The observed chromosome numbers would be related to some ecological factors (altitude, rainfall) of the origin environment of the populations. Chromosome measurements and established karyotypes were determined for the first time in *V. monardii*, *V. onobrychioides* and *V. ochroleuca subsp. atlantica*. Chromosome size and karyotype formula are variable among the studied species and subspecies. Karyotypes vary from symmetrical to asymmetrical and the intrachromosomal asymmetry is higher than interchromosomal one. The new cytogenetic data would contribute to a better understanding of the evolution mechanism of the species in the genus *Vicia* L.

Keywords: chromosomes, endemic, karyotype, plant genetic resources, *Vicia* L.

INTRODUCTION

The Leguminosae (*Fabaceae*) is the third largest angiosperm family in terms of species number after *Asteraceae* and *Orchidaceae,* comprising over 770 genera and 19500 species (Lewis et al. 2005, 2013). The family is morphologically, physiologically and ecologically diverse, representing one of the most spectacular examples of evolutionary diversification in plants (LPWG 2017).

The genus *Vicia* L. is a member of the legume tribe *Vicieae* of the subfamily *Papilionoideae* (Kupicha1976). The exact number of species in the genus *Vicia* L. is quite difficult to estimate, due to cytological and morphological differences (Kartal et al. 2020). It is including approximately 210 species that are widely distributed in temperate regions of Europe, Asia and America (Hanelt and Mettin 1989). Archeological evidence suggests that the main center of diversification of genus *Vicia* is the Mediterranean region (Raveendar et al. 2015). Most of species of the genus *Vicia* are annual but a few of them belonging to the section *Cracca* are perennial (Yamanoto 1973). Recently, the genus *Vicia* has been recognized for its vital role in sustainable agriculture (Han et al. 2021).

The most common classification is that of Kupicha (1976) revised by Maxted (1993). Kupicha (1976) divided the genus *Vicia* into two subgenera, *Vicilla* and *Vicia* with 17 and 5 sections, respectively. Subgenus *Vicia* is smaller than *Vicilla* but it is more coherent and includes the more agriculturally important species (Ruffini Castiglione et al. 2011). Maxted (1993) subdivided the subgenus *Vicia* into 9 series, 38 species, 14 subspecies and 22 varieties. The subgenus *Vicilla* is considered more primitive and diverse than subgenus *Vicia* (Kupicha 1976 and Maxed 1993).

According to Quezel and Santa (1962), the Algerian flora is represented by 26 species and 18 subspecies corresponding to the genus *Vicia* L. Two subspecies, *Vicia ochroleuca subsp. atlantica* and *Vicia ochroleuca subsp. baborensis* are respectively quite rare and very rare in Algeria. More recently, Dobignard and Chatelain (2012) report 39 taxa within the genus *Vicia* in Algeria, including two endemic subspecies, *V. ochroleuca subsp. atlantica* and *V. ochroleuca subsp. baborensis*. The prospecting and collection mission carried out, in 2016, in north-eastern Algeria, showed the frequency of *Vicia sativa*, followed by *Vicia disperma* and the rarity recorded in some encountred species such as *Vicia monardii, Vicia narbonensis* and *Vicia ochroleuca* (*subsp. atlantica*) (Issolah et al. 2022).

Vicia sativa L. is a variable genus comprising of several subspecies; it is most commonly called *Vicia sativa* complex (Cvs) or *sativa* aggregate (Jauzein 1995; Kartal et al. 2020; Benlioglu 2021). Members of this complex are morphologically, karyologically and ecologically variables and were considered to be in active evolution (Potokina et al. 2000; Shiran and Raina 2001; El-Bok et al. 2015), making identification difficult and confusing.

Several karyological studies have been carried out in *Vicia*. They had an important role in improvement and solving of several taxonomic problems between the related species (Lavia et al. 2009; Murti et al. 2012). There is a considerable variability in haploid nuclear DNA content (1.8-13.3 pg) and basic chromosome number (2n=10, 12 or 14) between *Vicia* species (Raina and Narayan 1984; Maxed 1995). This makes the genus an interesting model for the study of plant genome and Karyotype evolution (Navratilova et al. 2003). Most of them are diploids with a basic number x =5, 6 or 7 (Maxted 1991), while only six of them are polyploids (Cremonini et al. 1992).

In Algeria, karyological studies on spontaneous populations of the genus *Vicia* L. are very rare. Several species are totally unknown. The main objectives of this study are to determine the number and size of chromosomes within some species of the genus *Vicia* in Algeria, in order to contribute to a better understanding of the mechanism of evolution of the different species in this genus.

This work follows previous studies conducted on the evaluation and valorization of diversity in spontaneous and local fodder legumes in Algeria (Issolah and Abdelguerfi 1999; Bouziane et al. 2019; Chabouni et al. 2019; Issolah et al. 2006, 2012, 2015, 2018, 2022).

MATERIAL AND METHODS

Plant material

Following a prospecting mission carried out by INRAA through the North-East of Algeria (Issolah et al. 2022), several species belonging to the genus *Vicia* were collected. Seventeen (17) populations belonging to six (06) species of the genus *Vicia* L. (*V. sativa subsp. sativa* (04), *V. sativa subsp. macrocarpa* (03), *Vicia disperma* (03), *Vicia monardii* (04), *V. ohchroleuca subsp. atlantica* (01), *Vicia onobrychioides* (01) and *Vicia lutea* (01) were the subject of the present study (Table 1).

Mitotic study

Diploid chromosome numbers were counted from the seedling root tips which were germinated in Petri dishes on filter paper at room temperature. The root tips meristems (about 1 cm long; zone of active division)

Table 1. Ecological characteristics of natural habitats of several spontaneous populations within some *Vicia* L. species in Algeria.

Populations	Species	Subspecies	Origin	Altitude (m)	Rainfall (mm)
11/14	*V. sativa* L.	*subsp. sativa*	Boumerdes	25	850
65/15	*V. sativa* L.	*subsp. sativa*	Bejaia	450	950
75/15	*V. sativa* L.	*subsp.sativa*	Bejaia	550	950
63/16	*V. sativa* L.	*subsp.sativa*	Bejaia	160	950
55/14	*V. sativa* L.	*subsp.macrocarpa*	Bejaia	1250	1100
73/15	*V. sativa* L.	*subsp. macrocarpa*	Bejaia	860	700
77/15	*V. sativa* L.	*subsp. macrocarpa*	Bejaia	410	800
49/14	*V. disperma* DC.	-	Bejaia	550	700
80/14	*V. disperma* DC.	-	Bejaia	90	750
79/15	*V. disperma* DC.	-	Tizi-Ouzou	1030	1100
40/16	*V. monardiii* Boiss	-	Bouira	800	500
48/16	*V. monardiii* Boiss	-	Bejaia	560	700
51/16	*V. monardiii* Boiss	-	Bejaia	595	700
66/16	*V. monardiii* Boiss	-	Bouira	730	500
52/16	*V. ochroleuca* Spreng.	*subsp. atlantica*	Bejaia	1140	1100
82/14	*V. onobrychioides* L.	-	Tizi-Ouzou	750	950
83/15	*V. lutea* L.	*subsp..vestita*	Blida	230	650

Source: (Issolah et al. 2022, completed).

were excised in the morning between 7.30 am – 8.30 am periods of active cell division. Firstly, root tips were pretreated for 02 h with α- bromonaphtalene (1%) at room temperature. Then, they were fixed in ethanol chloroform acetic acid (6:3:1) during 24 h at 4 °C. Root tips were hydolysed with 1 N HCL and were stained using lactopropionic orcein solution (Dyer 1963).

Chromosome counts in mitosis metaphase and karyotype analyses were obtained usually based on five best plates (metaphase cells) of chromosomes, for each population.

Each chromosome was identified on the basis of its total chromosome length. Chromosomal nomenclature was carried out according to Levan et al. (1964). Idiograms were constructed by arranging the chromosomes in homologous pairs by order of their length.

For the numerical characterization of the karyotypes, the following parameters were calculated: The length of long arm (L), short arm(S), total chromosome length (LT= L+S) and the relative length (RL ‰) = 1000x TL/∑ TL (Levan et al. 1964).

To determine chromosome type and centromere position, two parameters were calculed: arm ratio (r = L/S) and centromeric index (CI%=S/LT x 100) according to the nomenclature of Levan et al. (1964).

To determine the asymmetry of the karyotype, the interchromosomal asymmetry was determined by calculating the coefficient of variation of chromosome length $\mathbf{CV_{CL}}$ (Paszko 2006), **Rec index** (Venora et al. 2002) and the interchromosomal asymmetry index $\mathbf{A_2}$ (Zarco 1986). The intrachromosomal asymmetry was determined by calculating, the mean centromeric asymmetry $\mathbf{M_{CA}}$ (Peruzzi and Eroglu 2013), the percentage of karyotype asymmetry index **AsK%** (Arano 1963), total form percentage **TF%** (Huziwara 1962), the **Syi** index (Greilhuber and Speta 1976), the intrachromosomal asymmetry index $\mathbf{A_1}$ (Zarco1986) and the degree of asymmetry of karyotype **A** (Watanabe et al. 1999).

Meiotic study

A meiotic analysis was conducted in order to confirm the results obtained during mitosis and determine the chromosome type of the newly observed chromosome in the considered Algerian populations (*Vicia* L.*)*. On this purpose, a trial was set-up at the INRAA experimental station. The protocol adopted is a total randomization. The sowing was carried out on 15 November 2018 at the rate of twenty seeds (individuals), for each population.

In April 2018, flower buds of different sizes were collected for each population and immediately fixed in a solution of Carnoy acetic ethanol (3:1 v/v). The hydrolysis was performed using 1N HCL for 1 to 3 min at 60°C. The staining was done with lactopropionic orcein at room temperature. The meiotic behavior was analyzed.

The observations of the mitotic plates were made using a Primo Zeiss Star microscope and photographed with a digital camera attached to this microscope. The

analysis of cytogenetic data was made with the Axiovision software (1999-2009). The different karyotype calculations were made with Excel (2007).

RESULTS

Mitotic analyses

In this study, the chromosome numbers and detailed chromosome measurements were determined for the natural populations of *Vicia species* in Algeria. Mitotic studies revealed that all studied populations were diploid (figure 1).

Vicia sativa subsp. sativa

At the metaphase stage, chromosomal counts revealed the existence of a chromosomal number (2n=2x=12) for all analysed populations of *Vicia sativa subsp. sativa*. However, a second chromosome number (2n=2x=10) was found in population 11/14 with a high frequency (80%) in the cells of the same individual and in different individuals of this population (figure1). This indicates a chromosomic variation within and between the populations of *Vicia sativa* L.

The shortest chromosome length is 1.9 µm (population n° 11/14); the longest one is 4.84 µm (population n° 63/16). The mean value of the total length TLG of all the studied populations is 3.65 µm. The centromeric index varies between 23.4 to 38.53 and relative lengths vary from 87.51 to 222.97. The chromosomes observed in *Vicia sativa subsp. sativa* populations were mainly submetacentric or subtelocentric types (Table 3).

For the karyotype asymmetry, the lowest value of intrachromosomal asymmetry M_{CA} is 30.63, the highest one is 47.63. The lowest value of interchromosomal asymmetry CV_{CL} is 9 and the highest value is 30 (Table 7).

The idiograms were drawn based on centromeric index and arranged in the decreasing size order (Figure 2).

Vicia sativa subsp. macrocarpa

The present study showed that 2n=2x=12 is the chromosome number of all populations of *Vicia sativa subsp. macrocarpa* (Table 2). The shortest chromosome length is 2.25 µm (population n° 77/15), the longest one is 4.85µm (population n° 55/14). The mean value of the total length TLG of all studied population is 3.46 µm, the centromeric index varies between 21, 2 and 37.92 and the relative lengths vary from 111 to 206.7 (Table 4).

For karyotype asymmetry, the lowest value for intrachromosomal asymmetry M_{CA} is 36.68, the highest one is 49.0. The lowest and highest values of interchromosomal asymmetry CV_{CL} are 13 and 20, respectively (Table 7).

The karyotype formula is very variable between populations of the same subspecies in *Vicia sativa* (Table 3, 4).

The idiograms of *Vicia sativa subsp. sativa* and *Vicia sativa subsp. macrocarpa* were illustrated on the basis of their centromeric index and arranged in descending order of the chromosomal size (Figure 2).

Vicia monardii

Within this rare species (Quezel and Santa 1962), our observations showed that populations n°51/16 and n°40/16 have a stable number of chromosomes (2n=14), while populations n°48/16 and n°66/16 have two chromosomes numbers (2n=12 and 2n=14) and (2n=14 and 2n=16), respectively (Table 2).

In *Vicia monardii,* karyological measurements were determined for the first time in the present study. The shortest chromosome length is 2.76 µm (population n°48/16), the longest chromosome is 6.37 µm (population n° 66/16). The mean value of the total length TLG of all studied population is 4.40. The centromeric index varies between 23.07 and 37.9 and the relative lengths vary from 87.45 to 196.29 (Table 5).

For the karyotype asymmetry, the lowest value of M_{CA} intrachromosomal asymmetry is 36, the highest one is 44. The lowest value for interchromosomal asymmetry CV_{CL} is 15.4 and the highest value is 24.22 (Table 7).

The karyotype formula is variable between the populations of *Vicia monardii*. The observed chromosomes are mainly submetacentrics (Table 5).

The idiograms were illustrated on the basis of the centromeric index and arranged in descending order of chromosome size (Figure 2).

Vicia ochroleuca subsp. atlantica

The somatic chromosome number of *Vicia ochroleuca subsp. atlantica* is 2n =2x=14 (Figure1). It is a new number observed for the first time in this endemic species in Algeria.

Karyological measurements were made for the first time within this subspecies. It is characterized by chromosomes with lengths of 3.19 - 4.86 µm and an average value of the total length of 4.14 µm. The centromeric

Table 2. Chromosome number within 17 Algerian populations of the genus *Vicia* L.

Population	Species	Subspecies	Chromosomes numbers	
			2n	n
11/14	*V. sativa*	*subsp. sativa*	10 and 12	5 and 6
65/15	*V. sativa*	*subsp. sativa*	12	6
75/15	*V. sativa*	*subsp. sativa*	12	6
63/16	*V. sativa*	*subsp. sativa*	12	6
55/14	*V. sativa*	*subsp. macrocarpa*	12	6
73/15	*V. sativa*	*subsp. macrocarpa*	12	6
77/15	*V. sativa*	*subsp. macrocarpa*	12	6
49/14	*V. disperma*	-	14	7
80/14	*V. disperma*	-	14	7
79/15	*V. disperma*	-	-	6 and 7
40/16	*V. monardii*	-	14	-
48/16	*V. monardii*	-	12 and 14	6 and7
51/16	*V. monardii*	-	14	-
66/16	*V. monardii*	-	14 and 16	7 and 8
52/16	*V. ochroleuca*	*subsp. atlantica*	14	-
82/14	*V. onobrychioides*	-	14	7
83/15	*V. lutea*	*subsp. vestita*	-	7

index varies from 25.92 to 38.91 and the relative length varies from 109.96 to 167.52 µm. The karyotype formula is 2n=14=2m+12sm (Table 6).

For the intrachmosomal asymmetry M_{CA} and inter-chromosomal asymmetry CV_{CL}, the values are 39.61 and 14.5, respectively (Table 7).

The idiogram was illustrated on the basis of the centromeric index and arranged in descending order of chromosome size (Figure 2).

Vicia onorychioides

For the polymorphic species *Vicia onobrychioides,* the somatic chromosome number *is* 2n =2x=14. It is a new number observed in this species (figure 1).

The shortest chromosome length, the longest chromosome length, the mean chromosome length and the total haploid length are 2.91, 4.55, 3.67and 25.69 µm, respectively.

The centromeric index varies from 22.63 to 38.14 µm, respectively. The relative length varies from113.27 to 177.11. The karyotype formula is 2n =14= 10sm+2m+2st (Table 6).

The values of intrachromosomal asymmetry M_{CA} and interchromosomal asymmetry Cv_{CL} are 39 and 16, respectively.

The idiogram was illustrated on the basis of the centromeric index and arranged in descending order of chromosome size (Figure 2).

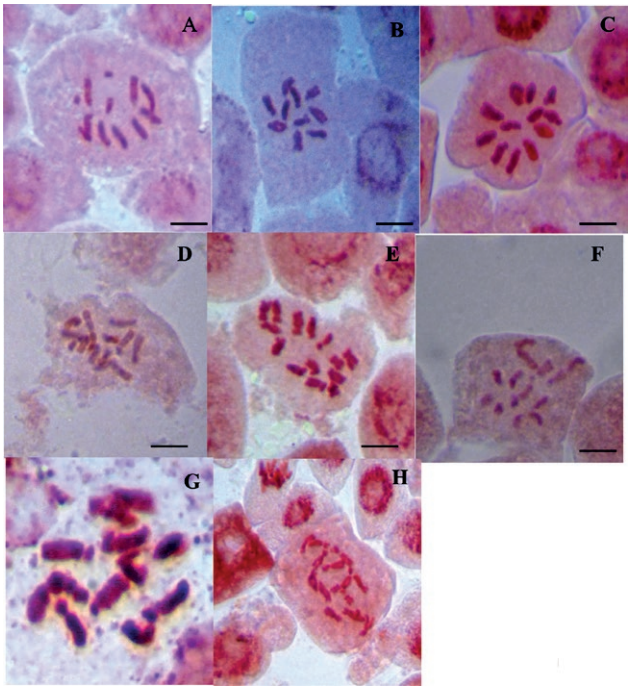

Figure 1. Mitosis observed in natural populations of the genus *Vicia* in Algeria*:* **A** - *Vicia sativa subsp. sativa* (population 11/14, 2n = 12) ; **B**- *Vicia sativa subsp. sativa* (population 11/14, 2n = 10); **C**- *Vicia sativa subsp. macrocarpa* (population 55/14, 2n = 12); **D**- *Vicia ochroleuca* (population 52/16, 2n = 14); **E**- *Vicia onobrychioides* (population 82/14, 2n = 14); **F**- *Vicia disperma* (population 49/15 , 2n= 14); **G** - *Vicia monardii* (population 48/16, 2n = 12); **H**- *Vicia monardii* (population 66/16, 2n = 16) . Scale bar 5 µm.

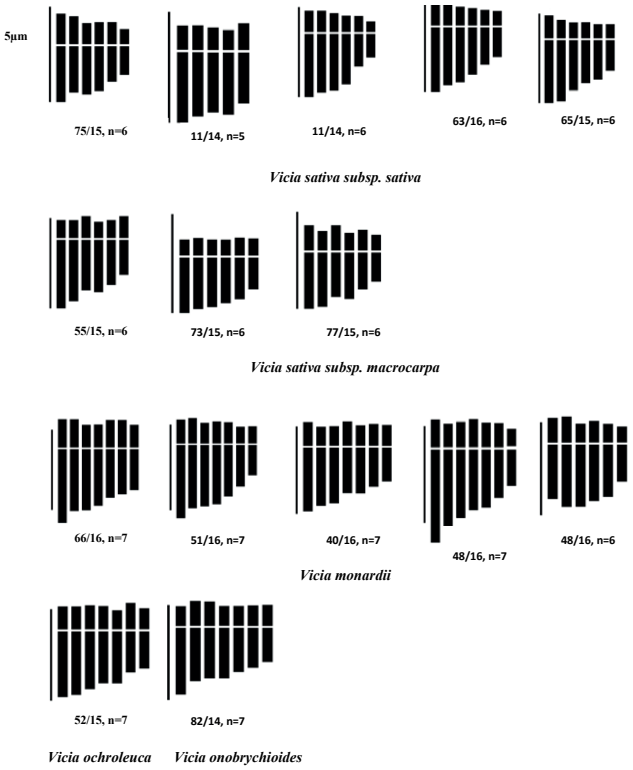

Figure 2. Idiograms of Algerian populations corresponding to four species in the genus *Vicia* L.

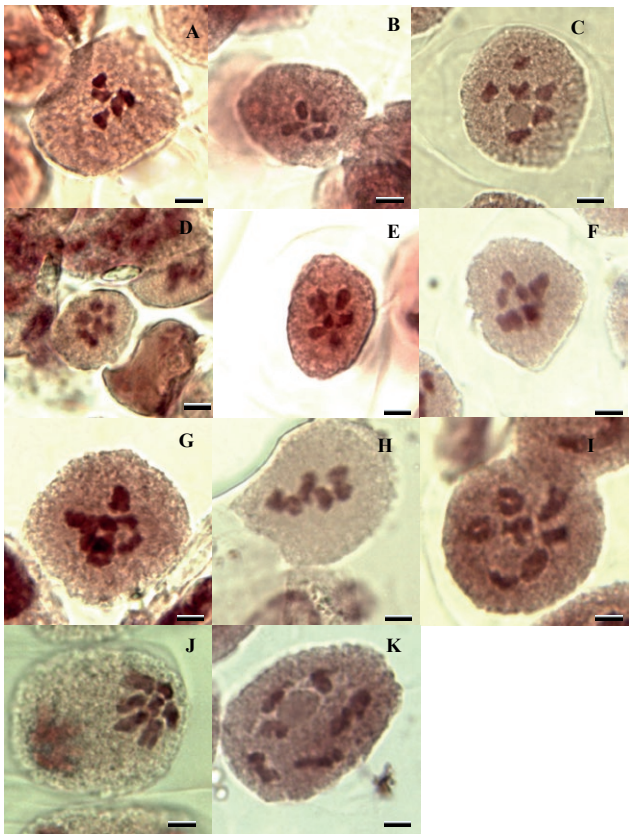

Figure 3. Meiosis observed in natural populations of the genus *Vicia* in Algeria. **A**- *Vicia sativa* subsp. *sativa* (population 11/14, n = 5); **B**- *Vicia sativa* subsp. *sativa* (population 11/14, n = 6); **C**- 55/14 *Vicia sativa* subsp. *macrocarpa* (population 55/14, n = 6); **D**- 48/16 *Vicia monardii* (population 48/16, n =7); **E**- *Vicia monardii* (population 48/16, n = 6); **F**- *Vicia monardii* (population 66/16, n = 7); **G**- *Vicia monardii* (population 66/16, n = 8); **H**- *Vicia disperma* (79/15 , n = 6); **I**- *Vicia disperma* (population 80/14, n = 7); **J**- *Vicia lutea* (population 83/15, n = 7); **K**- *Vicia onobrychioides* (population 82/14 , n = 7) . Scale bar 5 µ.

Vicia disperma

The somatic chromosome number of *Vicia disperma* is 2n =2x=14 for the populations 49/15 and 80/16 (Table 2).

DISCUSSION

The present study showed a high variation in the number, the asymmetry of chromosomes and the karyotype formula within Algerian populations of different species in the genus *Vicia* (*Vicia sativa, Vicia monardii, Vicia disperma, Vicia ochroleuca, Vicia onobrychioides and Vicia lutea).*

The chromosome number of *Vicia sativa. subsp. macrocapa* is 2n=12 for all Algerians populations. These results were in agreement with those of Raina and Rees (1983), Meriç and Dane (1999), Bisht et al. (1998), Raina et al. (2001), Basbag et al. (2013), Osman et al. (2020), Kartal et al. (2020) and Benlioglu (2021). However, Karyotype formula and quantitative analysis are variable among the populations, considered in the present study.

In *Vicia sativa subsp. sativa*, we recorded two chromosome numbers (2n=10 and 12). Similar results were reported by El-Bok et al. (2014) in Tunisian accessions of the same subspecies. According to Raina and Rees (1983), the multi basic chromosome number is a common phenomenon in the genus *Vicia* which was assigned as Rebertsonia translocation. The number (2n=12) has been reported by several authors in different ecotypes of *V. sativa subsp. sativa* (Meriç and Dane 1999; Navratilova et al. 2003; Gaffazardeh-Namazi et al. 2008; El-Bok et al. 2015; Martin et al. 2018; Osman et al. 2020). Ladizinsky and Shefer (1982) revealed that 2n=10 cytotypes were found in secondary and artificial habitats while 2n=12 cytotypes were found in natural vegetation among dwarf shrubs or in the maquis.

Within *Vicia sativa* aggregate, different experiments from several areas revealed that three chromo-

Table 3. The measurement data of chromosome pairs in Algerian populations of *V. sativa subsp. sativa.*

Pop	Pair	L(µm) (±SD)	S (µm) (±SD)	TL (µm)	ΣTL	TLG	RL$^0/_{00}$	r	Ci	Ct	Karyotype formula
	1	3.19(0.24)	1.08(0.26)	4.27			222.97	2.95	25.29	sm	
11/14	2	2.98(0.35)	1.1(0.26)	4.08			213.05	2.7	26.96	sm	
n=5	3	2.7(0.27)	1.07(0.16)	3.77	19.15	3.83	196.86	2.52	28.38	sm	4sm+1st
	4	2.74(0.29)	0.86(0.17)	3.6			188	3.18	23.88	st	
	5	2.29(0.24)	1.14(0.20)	3.43			179.11	2	33.32	sm	
11/14	1	3.5(0.37)	1.27(0.33)	4.77			219.71	2.75	26.62	sm	
n=6	2	3.24(0.11)	1.2(0.30)	4.44			204.51	2.7	27.02	sm	
	3	3.13(0.24)	1.05(0.23)	4.18	21.71	3.62	192.53	2.98	25.11	sm	
	4	2.8(0.61)	0.92(0.21)	3.72			171.34	3.04	24.73	st	5sm+1st
	5	1.8(0.66)	0.9(0.31)	2.7			124.36	2	33.33	sm	
	6	1.3(0.59)	0.6(0.06)	1.9			87.51	2.16	31.57	sm	
63/16	1	3.63(0.53)	1.21(0.11)	4.84			216.45	3	25	sm	
n=6	2	3.25(0.7)	1.18(0.22)	4.43			198.12	2.75	26.63	sm	
	3	3.08(0.32)	1.04(0.27)	4.12	22.36	3.73	184.25	2.96	25.24	sm	6sm
	4	2.65(0.50)	0.96(0.21)	3.61			161.44	2.76	26.59	sm	
	5	2.11(0.26)	0.86(0.17)	2.9			129.69	2.45	29.65	sm	
	6	1.68(0.30)	0.78(0.17)	2.46			110.01	2.15	31.7	sm	
65/15	1	3.49(0.62)	1.28(0.29)	4.77			220.53	2.72	26.83	sm	
n=6	2	3.39(0.69)	1.04(0.19)	4.43			204..8	3.25	23.47	st	
	3	2.83(0.65)	0.9(0.28)	3.73	21.63	3.61	172.44	3.14	24.12	st	4sm+2st
	4	2.37(0.62)	0.91(0.26)	3.28			151.64	2.6	27.74	sm	
	5	2.18(0.67)	0.77(0.12)	2.95			136.38	2.83	26.1	sm	
	6	1.69(0.54)	0.78(0.16)	2.47			114.19	2.16	31.57	sm	
75/15	1	2.92(0.51)	1.61(0.42)	4.53			217.68	1.81	35.54	sm	
n=6	2	2.43(0.21)	1.46(0.42)	3.89			186.92	1.66	37.53	m	
	3	2.53(0.36)	1.11(0.35)	3.64	20.81	3.47	174.91	2.27	30.49	sm	
	4	2.36(0.24)	1.16(0.26)	3.52			169.14	2.03	32.95	sm	4sm+2m
	5	1.85(0.11)	1.16(0.39)	3.01			144.64	1.59	38.53	m	
	6	1.47(0.20)	0.75(0.11)	2.22			106.68	1.96	33.78	sm	

Abreviations: long arm length (L), short arm length (S), total chromosome length (TL), mean value of total length (TLG), relative length (RL), arm ratio (r), centromeric index (Ci), chromosome type (Ct), median(m), submedian (sm), subterminal(st), standard deviation (SD).

some number (2n=10,12 and 14) were reported by several authors (Ladiznsky 1978; Yamanoto and Plitman 1980; Frediani et al. 2004; Arslan 2012; El -Bok et al. 2015; Martin et al. 2018; Kartal et al. 2020). However, the most reported chromosome number is 2n=12 (Ladizensky 1978; Ladizensky and Temkin 1978).

Very little work has been done on the species *Vicia monardii*. The present study follows and completes previous preliminary work (mitosis) carried out by our research team (Melzi 2018) on the same Algerian populations belonging to *Vicia monardii*. The preliminary results of mitosis (2n=12, 14 and 16) were confirmed through the present study and completed by the study of meiosis, indicating the effective presence of three chromosomes numbers (n=6, 7 and 8) in *Vicia monardii*, confirming that the observed chromosomes are indeed of type A.

Furthermore, in *Vicia sativa* and *Vicia monardii*, the chromosome numbers 2n=10 and 2n=12 were found only in populations originating from regions of low altitude and relatively high rainfall, whereas the number 2n=16 was found in populations originating from regions of high altitude and relatively low rainfall. Therefore, the variability of the chromosome number within and between the populations observed in *Vicia sativa* and *Vicia monardii*, would be linked to the ecological factors (altitude, rainfall) of the environment of origin of the considered populations.

In *Vicia onobrychioides* and the endemic subspecies *V. ochroleuca subsp. Atlantica,* the chromosome counts

Table 4. The measurement data of chromosome pairs in Algerian populations of *V. sativa subsp. macrocapa.*

Pop	Pair	L(µm) (±SD)	S(µm) (±SD)	TL (µm)	ΣTL	TLG	RL$^0/_{00}$	r	Ci	Ct	Karyotype formula
73/15	1	2.74(1.94)	0.78(0.13)	3.52			190.37	3.51	22.15	st	
n=6	2	2.6(0.62)	0.84(0.07)	3.44			186.04	3.09	24.41	st	
	3	2.44(0.52)	0.75(0.09)	3.19	18.49	3.08	172.52	3.25	23.51	st	2sm+4st
	4	2.26(0.31)	0.73(0.13)	2.99			161.7	3.09	24.41	st	
	5	2.1(0.57)	0.81(0.15)	2.91			157.38	2.59	27.83	sm	
	6	1.57(0.39)	0.87(0.23)	2.44			131.96	1.8	35.65	sm	
55/14	1	3.82(0.53)	1.03(0.33)	4.85			205.5	3.7	21.23	st	
n=6	2	3.38(0.15)	1.02(0.28)	4.4			186.44	3.31	23.18	st	
	3	2.8(0.42)	1.16(0.30)	3.96	23.6	3.93	167.79	2.41	29.29	sm	
	4	2.9(0.31)	0.94(0.15)	3.84			162.71	3.08	24.47	st	1m+2sm+3st
	5	2.45(0.38)	0.99(0.21)	3.44			145.76	2.47	28.77	sm	
	6	1.95(0.43)	1.16(0.38)	3.11			131.77	1.68	37.92	m	
77/15	1	2.94(0.50)	1.25(0.42)	4.19			206.7	2.35	29.83	sm	
n=6	2	2.82(0.25)	1.03(0.25)	3.85			189.93	2.73	26.75	sm	
	3	2.32(0.41)	1.34(0.36)	3.66	20.27	3.38	180.56	1.73	36.61	sm	6sm
	4	2.43(0.40)	0.94(0.16)	3.3			162.8	2.58	28.48	sm	
	5	1.96(0.41)	1.06(0.23)	3.02			148.99	1.84	35.1	sm	
	6	1.44(0.45)	0.81(0.11)	2.25			111	1.77	36	sm	

Abreviations: long arm length (L), short arm length (S), total chromosome length (TL), mean value of total length (TLG), relative length (RL), arm ratio (r), centromeric index (Ci), chromosome type (Ct), median(m), submedian (sm), subterminal(st), standard deviation (SD).

(mitoses) showed, for the first time, 2n=14. Bolkhoskik et al. (1974) reported a chromosome number of 2n =12 for *Vicia ochroleuca* and *Vicia onobrychioides*.

In the Algerian populations of *Vicia disperma* and *Vicia lutea*, the haploid chromosome number recorded is n= 7. This result is in agreement with the reports of Choii (1971) and El Allaoui- Faris (2011), concerning these same species. However, our study highlighted also a new number (n=6), reported for the first time in *Vicia disperma*.

The diploid number (2n =14) was recorded by several authors in *Vicia disperma* and *Vicia lutea* (Raina and Ress 1983; Jauzein1995; Venora et al. 2008; Bas Bag et al. 2013).

Through this study, we found that the number 2n=14 is more frequent in Algerian populations of the genus *Vicia* L. According to Holling and Satce (1974) and Tabour et al. (2002), 2n=14 is the most common chromosome number in the genus *Vicia* L. Thus, Raina and Ress (1983) indicated that the chromosome number 2n=14 is the most primitive in the genus *Vicia* and reported that the numbers 2n=10 and 2n=12 appeared later, by chromosomal rearrangement.

Concerning the chromosome size of the *Vicia sativa subspecies,* our results (1.9 - 4.84 µm) are very similar to those recorded by Benlioglu (2021) in Turkey (1.68-4.88 µm). However, they are relatively lower than the results reported by El-Bok et al. (2014) and Gaffazardeh-Namazi et al. (2008) on Tunisian (1.71 - 6 µm) and Iranian accessions (2.89 - 5. 69 µm) within the same subspecies.

Concerning the chromosome size observed in *V. sativa subsp. macrocarpa* (2.25 - 4.85 µm), it appears to be lower than that reported by Osman et al. (2020) (7.9 - 15.71 µm) and similar to that found by Benlioglu (2021), in some wild populations of the same species, in Turkey (2.54 - 4.98 µm).

Within the Algerian populations of the genus *Vicia* L., the chromosome size of the populations is relatively different from one species to another. According to their size, we can classify the chromosomes as follows: *Vicia sativa, Vicia onobrychioides, Vicia ochroleuca* and *Vicia monardii. Vicia sativa* presents the smallest chromosome size and *Vicia monardiii* is characterised by the largest one. Akpinar and Bilaloglu (1997) signalized that subspecies of *Vicia sativa* have smaller chromosomes and a lower DNA content than other species of the genus *Vicia*.

Exception made for *Vicia sativa*, detailed chromosome measurements and degrees of karyotype asymmetry, indicated through the present study, would be determined, for the first time, in some species (*Vicia monardii, Vicia ochroleuca and Vicia onobrychioides*).

Table 5. The measurement data of chromosome pairs in Algerian populations of *Vicia monardii.*

Pop	Pair	L(µm) (±SD)	S(µm) (±SD)	TL (µm)	ΣTL	TLG	RL$^0/_{00}$	r	Ci	Ct	Karyotype formula
66/16	1	4.60(1.00)	1.77(0.5)	6.37			179.43	2.59	27.78	sm	
n=7	2	3.86(0.89)	1.76(0.40)	5.62			185.3	2.19	31.31	sm	
	3	3.82(0.62)	1.42(0.26)	5.24			123.18	2.69	27.1	sm	
	4	3.54(0.81)	1.44(0.44)	4.98	35.5	5.07	144.68	2.45	28.91	sm	6sm+1m
	5	3.04(0.54)	1.72(0.30)	4.76			138.29	1.76	36.13	sm	
	6	2.82(0.70)	1.72(0.74)	4.54			131.9	1.63	37.88	m	
	7	2.56(0.53)	1.42(0.34)	3.98			115.63	1.8	35.67	sm	
51/16	1	4.26(0.86)	1.38(0.36)	5.64			184.07	3.08	24.46	st	
n=7	2	3.69(0.41)	1.48(0.32)	5.17			68.73	2.49	28.62	sm	
	3	3.56(0.47)	1.2 (0.25)	4.76			155.35	2.96	25.21	sm	
	4	3.44(0.46)	1.27(0.54)	4.71	30.6	4.37	153.72	2.70	26.96	sm	6sm+1st
	5	2.98(0.17)	1.23(0.22)	4.21			137.40.	2.42	29.21	sm	
	6	2.41(0.54)	0.98(0.27)	3.39			110.63	2.45	28.9	sm	
	7	1.76(0.31)	1.00(0.09)	2.76			90.08	1.76	36.23	sm	
40/16	1	3.66(0.54)	1.35(0.49)	5.01			177.91	2.71	26.94	sm	
n=7	2	3.34(0.78)	1.08(0.20)	4.42			156.69	3.09	24.43	st	
	3	3.20(0.56)	1.11(0.15)	4.31			153.05	2.88	25.75	sm	
	4	2.62(0.6)	1.38(0.60)	4.00	28.2	4.02	142.04	1.89	34.5	sm	5sm+1m+1st
	5	2.63(0.32)	1.16(0.34)	3.79			134.58	2.26	30.6	sm	
	6	2.25(0.68)	1.24(0.13)	3.49			123.93	1.81	35.53	sm	
	7	1.95(0.28)	1.19(0.14)	3.14			111.5	1.63	37.9	m	
48/16	1	4.80(0.61)	1.44(0.04)	6.24			196.29	3.33	23.07	st	
n=7	2	4.01(0.56)	1.23(0.03)	5.24			164.83	3.26	23.47	st	
	3	3.53(0.47)	1.33(0.28)	4.86			152.88	2.65	27.36	sm	
	4	3.13(0.06)	1.46(0.12)	4.59	31.8	4.54	144.38	2.14	31.8	sm	5sm+2st
	5	2.99(0.34)	1.29(0.23)	4.28			134.63	2.31	30.14	sm	
	6	2.51(0.33)	1.27(0.13)	3.80			119.53	1.97	33.42	sm	
	7	1.88(0.87)	0.90(0.28)	2.78			87.45	2.08	32.37	sm	
48/16	1	2.92(0.25)	1.33(0.25)	4.23			174.36	2.18	31.44	sm	
n=6	2	3.35(0.37)	1.41(0.21)	4.76			196.20	2.37	29.62	sm	
	3	3.34(0.27)	1.03(0,13)	4.37	24.3	4.04	180.13	3.24	23.56	st	5sm+1st
	4	3.03(0.15)	1.17(0.05)	4.2			173.12	2.58	27.85	sm	
	5	2.82(0.08)	1.02(0.18)	3.84			158.28	2.76	26.56	sm	
	6	2.00(0.50)	0.86(0.07)	2.86			117.88	2.32	30.07	sm	

Abreviations: long arm length (L), short arm length (S), total chromosome length (TL), mean value of total length (TLG), relative length (RL), arm ratio (r), centromeric index (Ci), chromosome type (Ct), median(m), submedian (sm), subterminal (st), standard deviation (SD).

The presence of different karyotypes formulas within Algerians populations of the genus *Vicia* may be due to the ecological differences characterizing their geographic origins. According to Benlioglu (2021), the changes in the structure of chromosome morphology can be explained as a gradual alteration which occurred through the evolution of the karyotype during natural or manual selection.

In *Vicia sativa subsp. sativa*, the karyotype formulas reported in the present study, are different from those reported by other authors: 1m +5 st (Namazi et al. 2008; El- Bok et al. 2014), 1m+1sm+3st (El -Bok et al. 2014), 3 m +3st (Osman et al. 2020), 3m+3sm, 2m+3sm and 2sm+4st (Benlioglu 2021), but similar (5sm+1st) to that reported by Martin et al. (2018).

The chromosomes observed in Algerian populations of *genus Vicia* were mainly submetacentric. According to Zuo and Yuan (2011), the predominance of submetacentric chromosomes indicated that these populations might have retained some of their primitive wild traits.

Table 6. The measurement data of chromosomes pairs in Algerian populations of *Vicia onobrychioides* and *Vicia ochroleuca subsp. atlantica.*

Pop	Species		BL(µm) (±SD)	BC(µm) (±SD)	LT	∑LT	TLG	$RL^0/_{00}$	R	Ci	Ct	Karyotype formula
52/16		1	3.60(0.58)	1.26(0.19)	4.86			167.52	2.85	25.92	sm	
n=7		2	3.48(0.63)	1.26(0.05)	4.74			163.39	2.76	26.58	sm	
		3	3.17(0.69)	1.32(0.27)	4.49			154.77	2.4	29.39	sm	
	V. ochroleuca subsp. atlantica	4	2.85(0.38)	1.27(0.24)	4.12	29.01	4.14	142.01	2.24	30.82	sm	6sm+1m
		5	2.86(0.51)	1.05(0.20)	3.91			134.78	2.72	26.85	sm	
		6	2.26(0.43)	1.44(0.17)	3.70			127.54	1.56	38.91	m	
		7	2.03(0.27)	1.6(0.09)	3.19			109.96	1.75	36.36	sm	
82/14		1	3.52(0.82)	1.03(0.28)	4.55			177.11	3.41	22.63	st	
n=7		2	2.81(0.42)	1.3(0.30)	4.11			159.98	2.16	31.63	sm	
		3	2.66(0.34)	1.28(0.17)	3.94			153.36	2.07	32.48	sm	
	V. onobrychioides	4	2.66(0.38)	1.04(0.21)	3.70	25.69	3.67	144.02	2.55	28.10	sm	5sm+1m+1st
		5	2.33(0.21)	1.04(0.20)	3.30			128.45	2.24	31.51	sm	
		6	2.10(0.21)	1.08(0.11)	3.18			123.97	1.94	33.96	sm	
		7	1.8(0.18)	1.11(0.15)	2.91			113.27	1.62	38.14	m	

Abreviations: long arm length (L), short arm length (S), total chromosome length (TL), mean value of total length (TLG), relative length (RL), arm ratio (r), centromeric index (Ci), chromosome type (Ct), median(m), submedian (sm), subterminal (st), standard deviation (SD).

Table 7. The asymmetry index values in natural populations of *Vicia* species in Algeria.

Pop	Species	2N	Ask	TF	Syi	Rec	A_1	A_2	A	CV_{cl}	M_{CA}
11/14	*V. sativa subsp. sativa*	10	72.58	27.41	37.77	89.69	0.47	0.09	0.45	9	45.16
11/14	*V. sativa subsp. sativa*	12	72.63	27,36	37.8	75.85	0.15	0.3	0.45	30	45.02
63/16	*V. sativa subsp. sativa*	12	73.34	26.97	36.81	76.99	0.14	0.24	0.46	24	46.38
65/15	*V. sativa subsp. sativa*	12	73.74	26.25	35.47	75.57	0.53	0.24	0.47	24	47.63
75/15	*V. sativa subsp. sativa*	12	65.16	34.83	53.09	76.56	0.4	0.22	0.3	22	30.63
55/14	*V. sativa subsp. macrocarpa*	12	73.3	26.69	36.45	81.09	0.47	0.16	0.46	16	45.56
73/15	*V. sativa subsp. macrocarpa*	12	74.14	21.14	34.21	87.55	0.61	0.13	0.49	13	49.01
77/15	*V. sativa subsp. macrocarpa*	12	68.62	31.72	46.32	80.62	0.46	0.2	0.36	20	36.68
40/16	*V. monardii*	14	69.77	30.22	43.21	78.88	0.39	0.15	0.39	15.40	39
48/16	*V. monardii*	12	71.88	28.11	38.96	84.94	0.43	0.16	0.44	16.00	44
48/16	*V. monardii*	14	71.87	28.05	38.95	72.77	0.86	0.24	0.44	24.22	44
51/16	*V. monardii*	14	72.12	27.87	38.73	77.60	0.39	0.23	0.44	23.00	44
66/16	*V. monardii*	14	68.44	31.7	46.1	79.61	0.20	0.16	0.36	16.40	36
82/14	*V. onobrychioides*	14	69.59	30.67	43.92	80.65	0.44	0.16	0.39	16	39
52/16	*V. ochroleuca subsp. atlantica*	14	69.8	30.19	43.25	85.27	0.37	0.14	0.39	14.5	39.61

Abreviations: karyotype asymmtry index(Ask), total form percentage (TF), the index of karyotype symmetry (Syi), the symmetric index (Rec), the intrachromosomal asymmetry index (A_1), interchromosomal asymmetry index(A_2), degree of asymmetry of karyotype (A), coefficient of variation of the centromeric index (CV_{CL}), mean centromeric asymmetry (M_{CA}).

The predominance of subtelocentric chromosomes in *V. sativa subsp. macrocarpa* populations (73/15 and 55/14) indicated asymmetrical Karyotypes. According to Hanelt and Mettin (1989), the subtelocentric chromosomes are predominant in the subgenus *Vicia* and it may have an evolutionary significance.

All the chromosomes of the population 77/15 coresponding to *V.sativa subsp. macrocarpa* are sub median. According to Paszko (2006), the karyotype of the last population *V. sativa subsp. macrocarpa* is considered as symmetrical.

Several authors (Maxed et al. 1991; Kamel 1999; Weber and Shifino-Wittman 1999; Navratilova et al. 2003) demonstrated that *Vicia sativa subsp. sativa* is the only subspecies of *Vicia sativa* that has a metacentric pair in its Karyotype. However, our study showed

the presence of one pair of metacentric chromosomes in *Vicia sativa subsp. macrocarpa* (population n° 55/14). This type of chromosome is also indicated by El Bok et al (2014), Osman et al. (2020) with 3m+2sm+1st and Benlioghlu (2021) with 4sm+2st and 3 m +3sm.

In *Vicia monardii*, the symmetrical karyotype is represented by the population 66/16, while asymmetrical karyotypes characterize the populations 40/16, 48/16 and 51/16 with a predominance of subtelocentric chromosomes.

The study of karyotype asymmetry is one of the most important parameters in the karyomorphology (Astuti et al. 2017; Shamsolshoara et al. 2020; Martin et al. 2018). The intrachromosomal asymmetry gradually increases with centromere shift from median point to terminal point, while the interchromosomal asymmetry increases with more chromosome size heterogeneity (Martin et al. 2018).

In the present study, karyotype asymmetry was assessed on the basis of quantitative indices. The values of some indices (M_{CA}, $CV_{cl,}$ Ask %, A_1, A_2 and A) increase with increasing asymmetry while the values of some indices (TF%, Syi and Rec) decrease with increasing asymmetry (Zuo and Yuan 2011; Eroglu et al. 2013; Atlay et al. 2017).

Based on the following asymmetry indices: M_{CA}, AsK, TF, Syi and A, the population 75/15 (*V. sativa ssp. sativa*) presents the most symmetrical karyotype, while the population 73/15 (*V. sativa subsp. macrocarpa*) is characterized by the most asymmetrical one. However, the asymmetric karyotypes are different in interchromosomal asymmetries. The population 11/14 (2n=10) (*V. sativa subsp. sativa*) is the most symmetrical karyotype with respect to three indices (CV_{CL}, Rec and A2), whereas the population 48/16 (2n=14) corresponding to *Vicia monardii* species, is the most asymmetrical karyotype, with respect to only two indices (Rec and A_2).

The results of the karyotype asymmetry index analysis showed that the chromosomes of the studied species in the genus *Vicia* vary from median to subterminal. The karyotypes vary from symmetrical to asymmetrical and the intrachromosomal asymmetry was higher than interchromosomal one. These results were in agreement with the reports of Martin et al. (2018) and Benlioglu (2021)**,** within the genus *Vicia***.**

According to Kamel et al. (1999), the evolution of Karyotype might be inferred from symmetry to asymmetry as a result of pericentric inversion or unequal translocation.

The differences in the asymmetry of karyotype were great in the genus *Vicia*, for which it may be assumed that diversity of the genus has been accompanied by very small changes in the structure of the chromosome (El-Bok et al. 2014). According to Altay et al. (2007), the difference in chromosome morphologies may contribute to the variation of the genera, sections and species.

In addition, a number of studies have been carried out, using different methodological approaches to gain a better understanding of the complex phylogenetic relationships between the different species of the *Vicia* genus.

Thus, our results indicated that x = 7 is the most frequently observed number in the six *Vicia* species. Previous work has confirmed that x = 7 is the ancestral number of the genus *Vicia* (Shiran et al. 2014). For their part, Metin and Hanelt (1964) hypothesised that x = 7 is the most likely chromosome number in the genus *Vicia* and the numbers x= 6 and x= 5, observed in some species, are derived base numbers. Other authors think that x = 5 is the basic number and x = 6 and 7 are derived numbers (Schubert et al. 1986).

From a phylogenetic point of view, the results of Schaefer et al. (2012) showed that the phylogenetic relationships between species in the genus *Vicia* are as follows: In section *Cracca*, *Vicia disperma* is closely related to *Vicia ochroleuca* and *Vicia monardii*, but the degree of relationship between *Vicia disperma* and *Vicia ochroleuca* is less than that found between *Vicia disperma* and *Vicia monardii*; these two species (*Vicia disperma* and *Vicia monardi*) are the most closely related species.

In the *Sativa* section, *Vicia sativa* is distant from *Vicia lutea*, but it is closely related to *Vicia angustifolia*. On the other hand, *Vicia onobrychioides* in the *Pedunculatea* section is distant from all the other studied species in the *Cracca* and *Vicia* sections (Schaefer et al. 2012).

According to Shiran et al. (2014), *V. sativa subsp. sativa* is distant from *V. sativa subsp. macrocarpa* but it is closely related to *V. sativa subsp. angustifolia* and these two subspecies (*subsp. sativa and subsp. macrocarpa*) are distant from *Vicia lutea* and *Vicia disperma*.

Shiran and Raina (2001) and Shiran et al. (2014) revealed that within the *Vicia sativa* complex two lineages are evident in all phylograms. Lineage 1 consists of *V. sativa subsp. macrocarpa* and *V. sativa subsp. angustifolia*, while lineage 2 includes *V. sativa subsp. sativa*, *V. sativa subsp. cordata, V. sativa subsp. amphicarpa, V. sativa subsp. incisa and V. sativa subp. nigra* (Shiran and Raina 2001; Shiran et al. 2014).

More recently, results have described a close relationship between *Vicia macrocarpa* and *Vicia narbonensis* (Osman et al. 2020).

CONCLUSION

The present study highlighted the characteristics of the chromosomes in the natural populations belonging

to six (06) species (*Vicia sativa*, *Vicia disperma*, *Vicia monardii*, *Vicia ohchroleuca*, *Vicia onobrychioides* and *Vicia lutea*) of the genus *Vicia,* coming from different eco-geographical zones of North-Eastern Algeria, and the relationships which would be exist with some ecological factors of the environment of origin (altitude and rainfall).

The results carried out in mitosis and meiosis, showed the presence of some new chromosomal numbers in Algerian populations of the genus *Vicia* L. The first number (2n=14, n=7) was observed for the first time in the endemic subspecies *Vicia ochroleuca subsp. atlantica* (Population n° 52/16) and the species *Vicia onobrychioides* (Population n° 82/14). The second number (n=6) was observed in *Vicia disperma* (Population n° 79/15).

In *Vicia monardii*, the three new numbers of chromosomes previously observed in mitosis (2n=12, 14 and 16), were confirmed through the study of meiosis (n=6, 7 and 8), indicating that they are indeed A-type chromosomes.

This study has shown that the base number x=7 is the most frequently observed number in the six *Vicia* species.

The observations showed that chromosome numbers 2n=10 and 2n=12 are more frequently encountered within populations located in regions of relatively low altitude and relatively high rainfall, respectively in the two species *Vicia sativa* and *Vicia monardii*. The number 2n =16 is only found in populations of *Vicia monardii* originating from high altitude and relatively low rainfall regions.

Exeption made for *Vicia sativa*, detailed chromosome measurements and degrees of karyotype asymmetry would be determined for the first time in the following species: *Vicia monardii, Vicia ochroleuca and Vicia onobrychioides.*

The karyological variations observed in Algerian populations corresponding to some species of the genus V*icia* are clearly detectable in the chromosomal morphologies. The chromosomes vary from median to subterminal and the karyotype varies from symmetrical to asymmetrical. Ecological conditions, in particular the altitude factor of the geographical origin of the populations, would have an effect on the changes in chromosome structure.

This research is a contribution to the evaluation and valorization of plant genetic resources in Algeria, particularly in the genus *Vicia* L. The analysis of chromosomal diversity, based on new data, allowed to answer some questions related to the mechanism of evolution of the species belonging to the genus *Vicia* L. The characterization carried out could play an important role in the conservation and use of these genetic resources through a plant breeding programme.

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Cytogenotoxic and antimicrobial effects of *Nezara viridula* (L.) (Hemiptera: Heteroptera: Pentatomidae) alcoholic extracts

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Abstract. Due to their multifunctionality and the numerous fields of applicability, insects are extensively studied today for both their biomedical and nutritional properties. In the current study the cytogenotoxic and antimicrobial potential of ethanol and methanol extracts of *Nezara viridula* (Linnaeus 1758) was evaluated using the *Allium* test, respectively the disk diffusion test. A mitostimulatory effect of the extracts of *N. viridula* and a variation of the cytogenotoxic activity of the extracts in a gender-dependent manner was noticed. As well, significant variations of the mitotic index were determined through the type of solvent used and the concentration of the extracts. High frequency chromosomal aberrations and mitotic abnormalities were recorded with high concentration ethanolic extracts. Following the testing of four standard bacterial strains and two standard yeast strains, a slightly antimicrobial activity was observed when compared to control. The use of invasive species in such studies opens up new perspectives on the potential of organisms considered harmful.

Keywords: insect, gender, extracts, bioactivity, mitotic index.

INTRODUCTION

Throughout time, despite their numbers and their significant therapeutic properties, insects had a minor role in traditional medicine and healthcare practices or in the synthesis of modern drugs as compared to plants. In traditional medicine the use of insect species has been recorded in regions of eastern Asia (e.g. China, India, Korea, and Japan), Africa, South and Central America (Costa-Neto 2002; Figueirêdo et al. 2015; Meyer-Rochow 2017; Bairagi 2019; Zhang et al. 2023; Yong et al. 2023).

According to Feng et al. (2009), in China, over 100 insect species have been used for their medicinal potential since ancient times. Namba et al. (1988) showed that 54 types of crude drugs derived from insects are men-

tioned in a Chinese manuscript from the beginning of the 7th century. Stink bugs are among the insect species mentioned by traditional medicine. *Aspongopus chinensis* (Hemiptera: Pentatomidae), common in China and known in traditional medicine for its analgesic effects and for its role in the treatment of nephropathy, was investigated recently for its antitumor properties (Luo et al. 2012; Tan et al. 2019). Syrup, powder, wax, oils, and tea obtained from the eggs, larvae or adults of the insects from the families Formicidae, Belastomatidae, Termitidae, Cicadidae, Gryllotalpidae, Asilidae, Pompilidae, Pentatomidae, etc. are used in north-east of Brazil for therapeutic purposes (Costa-Neto 2002).

In the European culture, the use of insects as a source of food or for therapeutic purposes is rarely mentioned (Ulicsni et al. 2016). Cantharidin, known in European traditional medicine especially for its high toxicity to human body, has anti-tumor properties (Rauh et al. 2007). Maggot therapy, simple and effective, has been used in Europe in the treatment of chronic wounds, such as diabetic food wounds or postoperative infections (Sherman et al. 2000). The use of the products provided by *Apis mellifera*, such as honey, venom, royal jelly, and propolis, was discussed in recent, comprehensive reviews (Pasupuleti et al. 2017; Wehbe et al. 2019).

The reviews by Zhou et al. (2005) and Park and Kim (2010) systematized the notable applications of chitin and its derivatives, which due to their biocompatibility and non-toxic nature were thoroughly studied to document their biological and biomedical properties. Furthermore, an increasing number of authors have observed the progress made during the last decades in the treatment of different conditions through the use of compounds obtained from insects and other arthropods. They support the development of insect-based biotechnologies and biotesting using insects in order to obtain new products for modern medicine (Ratcliffe et al. 2014; Ejiofor 2016; Seabrooks and Hu 2017). Ratcliffe et al. (2011) recommended the use of insects as models in the study of the immune response to human pathogens.

Moreover, in a world facing an alarming increase in bacterial and fungal resistance to antimicrobials, the identification of new substances or complex mixtures with antimicrobial properties has become a priority. For instance, strains of *Staphylococcus aureus* have a great variety in their resistance to antibiotics, often through horizontal gene transfer of genetic elements (Foster 2017), so new therapeutic solutions are required. *Candida albicans* and *C. parapsilosis* are human pathogens and also a normal commensal; the frequency of *C. parapsilosis* infections is higher in immunocompromised patients (Trofa et al. 2008) and they need an alternative solution for treatment.

The green stink bug *N. viridula* presents a remarkable polyphagia, with over 150 plant species identified as hosts; however, they prefer leguminous and brassicaceous plants and they cause serious damage to these plants (Oho and Kiritani 1960; Panizzi et al. 2000; Panizzi 2004). The very efficient secretory / defensive system developed by this species was probably one of the most important factors that led to their worldwide range expansion. A series of research studies was focused on the composition and the role of secretions in stink bugs (Gilby and Waterhouse 1965; Aldrich et al. 1978; Lockwood and Story 1987; Borges and Aldrich 1992; Pavis et al. 1994; Sturaro et al. 1994).

Starting from the wide distribution of the species *N. viridula* and the fact that it is an invasive species with specific defensive secretions, in the current paper we set out to investigate the cytogenotoxic and antimicrobial effects of alcoholic extracts of *N. viridula* as a platform for further applications. From our knowledge, this is the first paper to present the bioactivity of alcoholic extracts of *N. viridula*.

MATERIALS AND METHODS

Preparation of insect extracts

The biological material consisted of larvae and adults of *N. viridula* collected in the period July-October from cucumbers of the Cornichon variety from an organic culture located in the Cotmeana Plateau in southern Romania, from site N 44.98482°, E 024.72828°, altitude 348 m a.s.l. The larvae were reared in laboratory conditions until the emergence of adults; they were fed with organic cucumber leaves. The two genders were separated considering the phenotypic differences and the biological material was kept in the freezer at -18 °C until the preparation of extracts. The alcoholic extracts of *N. viridula* were obtained by grinding and macerating 5 g of each gender, females and males respectively, in 100 ml ethyl alcohol 96° and methyl alcohol 96°, respectively, for 48 hours, at room temperature (18-20 °C). The extracts were filtered using Whatman no. 1 filter paper.

Evaluation of genotoxic activity of male and female extracts

The cytogenotoxic effects of the *N. viridula* extracts were evaluated with the *Allium* test. The bulbs of *A. cepa* (a local variety) with a diameter of 3-4 cm were taken from a private farm. No phytosanitary treatments were applied to obtain the bulbs. Before use they were macro-

Table 1. Encoding of the samples.

Encoding	Male/Female (M/F)	Ethanol/Methanol (Et/Met)
NEM 5% / 15% / 25%	M	Et
NMM 5% / 15% / 25%	M	Met
NEF 5% / 15% / 25%	F	Et
NMF 5% / 15% / 25%	F	Met

scopically inspected to be free from pests. Root primordia were carefully exposed and immediately afterwards the bulbs were suspended in 30 ml containers with the discoid stem in contact with distilled water for 48 h. The treatment with ethanol and methanol female and male extracts of *N. viridula* with concentrations of 5%, 15% and 25% (Table 1) was applied for the next 48 h. The negative sample was represented by the onion roots obtained by suspending the bulbs with the discoid stem in contact with distilled water for 96 hours. Three identical samples were prepared for each specific sample configuration. Rhizogenesis was stimulated by keeping the containers in the dark at room temperature (20-22 °C).

After 96 hours of semi-static exposure (Rank 2003), the roots with a length of 5-10 mm were cut from the base with a sharp razor blade and immersed into Framer's fixative (absolute ethanol: glacial acetic acid, 3:1 v/v), over the night, at 4 °C, to preserve cell integrity. The fixed roots were hydrolysed in HCl 1N, at 60 °C, for 15 minutes by partial dissolution of pectic substances and stained with orcein-acetic solution 1%, for 15 minutes, at 60 °C. Microscopic slides were obtained through the squash technique. To prevent the quick drying of the microscopic slides the edges of the cover slips were sealed with nail varnish (Grant 1982).

Microscopic slides were analysed using an Olympus CX 31 microscope at a magnification of 400× (ocular - objective 10×40). The representative images of the different phases of mitosis, as well as of the chromosomal aberrations were captured using Color View I CCD digital camera.

For each experimental sample we analysed approximately 3 000 cells in different phases of the cell cycle. The mitotic index (MI) was determined as the percentage ratio of the total number of mitotic cells to the total number of cells examined in the microscopic preparation. The frequency of mitotic phases was determined by calculating the percentage ratio of the number of cells in a certain mitotic phase (prophase, metaphase, anaphase or telophase) to the total number of cells examined in the microscopic slides.

Evaluation of antimicrobial effect of insect extracts

To estimate the antimicrobial effect of insect extracts we used four standard bacterial strains (both Gram positive and Gram negative): *Staphylococcus aureus* ATCC 25923, *Streptococcus pyogenes* A Group ATCC 19615, *Bacillus subtilis* subsp. *spizizenii* ISM 68/53 (equivalent ATCC 6633) and *Escherichia coli* ATCC 25922. Two standard yeast strains of *Candida albicans* ATCC 10231 and *C. parapsilosis* ATCC 22019 were also used.

In vitro assessment of antimicrobial effects of *N. viridula* extracts were performed by disk diffusion test (Ma et al. 2019; Balouiri et al. 2016). Specific culture medium was used to test the sensitivity of standard bacterial strains: Mueller Hinton agar (MHA) for *S. aureus*, *B. subtilis* and E. *coli* strains, Mueller Hinton agar supplemented with 5% for *S. pyogenes* strain and Sabouraud agar for *Candida* strains (Graso Biotech).

The bacterial or yeast suspensions (0.5 McFarland) were inoculated on the sterile medium, then sterile filter paper disks (6 mm Ø) were placed onto medium surface. Each paper disk was impregnated with 10 µL of undiluted insect extracts. Ethanol 96° (E) and methanol 99.8% (M) were used as negative controls. Gentamicin 10 µg per disk (Tody Laboratories) and Fluconazole 25 µg per disk (Oxoid) were used as positive controls to test bacteria (ATB), and yeasts (AM), respectively. The experimental variants were coded according to Table 1. After 18-20 h at appropriate temperature (37 °C), the diameter of inhibition zones was measured and the average of those three values was compared. The yeast strains were incubated for 48 h to reveal the sensitivity to the extracts.

Statistical analysis

Each experimental variant comprised 3 trials. For the processing and valorisation of the data we used the statistical analysis program SPSS for Windows (Statistical Package for Social Science), version 20.0 (2010), applying the One-Way ANOVA model, and the Duncan's test for multiple comparison, respectively. The significance of the differences between the effects of the variables or the interaction between them, for which the calculated F had significant values at a level of confidence of 95%, was noted in small letters. The relationship between an interval variable and a categorical variable was determined by Eta correlation ratio. The results are presented as mean ± SE for n=3 bulbs/ sample.

RESULTS

Cytogenotoxic activity of male and female extracts of N. viridula

After 48 hours from the exposure to the alcoholic extracts, the cells in different mitotic phases were evaluated using the optical microscope; results are shown in Figure 1 as percentage of cells in mitosis. The MI determined for the control sample (5.96%) was not significantly different from the MIs calculated for the samples defined by the concentration 5% of the extracts of *N. viridula*. Similarly, compared to the control, the extracts of *N. viridula* with a concentration of 15% produced an insignificant increase of the MI, except for NEF 15%. However, increasing the concentration of the extracts to 25% was associated with a significant increase of MI in meristematic root cells, irrespective of the solvent used.

Regarding the correlation between the dependent and the nominal variables, the Eta coefficient of 0.638 indicates that 63% of the IM variation may be attributed to the independent variable (concentration), while the Eta coefficient of 0.09 indicates that there is a very slight positive correlation between gender and IM, with only 0.09% of the IM variation being attributed to gender (Table 2).

Figure 2 shows the results regarding the distribution of the phases of mitotic division in the root meristem cells of *A. cepa* exposed to the action of ethanol and methanol female and male extracts of *N. viridula.*

Table 2. Influence of nominal variables on the mitotic index in the root meristem cells of *A. cepa* (interpretation of the correlation between the dependent and the categorical variables using the Eta coefficient).

Directional measures		Value
Nominal by Interval Eta	Concentration Dependent	1.000
	IM Dependent	0.638
	Gender Dependent	0.957
	IM Dependent	0.098

In the control roots the values of the indices were of 75.4% for the prophase, 11.2% for the metaphase, 4.7% for the anaphase, and 8.6% for the telophase. Keeping the roots for 48 hours in ethanol and methanol extracts of *N. viridula* with a concentration of 5% led to a significant increase in the percentage of prophases associated with a significant decrease of metaphases, except for the sample NMM 5%.

The prophase index with a high value was associated with decreased or zero metaphase index. A significant increased metaphase index was noticed for NMM 5%, 15% and 25%. Compared with the control, the highest values of the anaphase index were recorded in the experimental samples NEF 15%, NEM 25% and NMM 25% (Figure 2).

The genotoxic effects of the alcoholic extracts of *N. viridula* were assessed by registering the chromosomal aberrations in the meristematic root cells of *A. cepa* (Table 3). Sticky chromosomes, anaphase bridges,

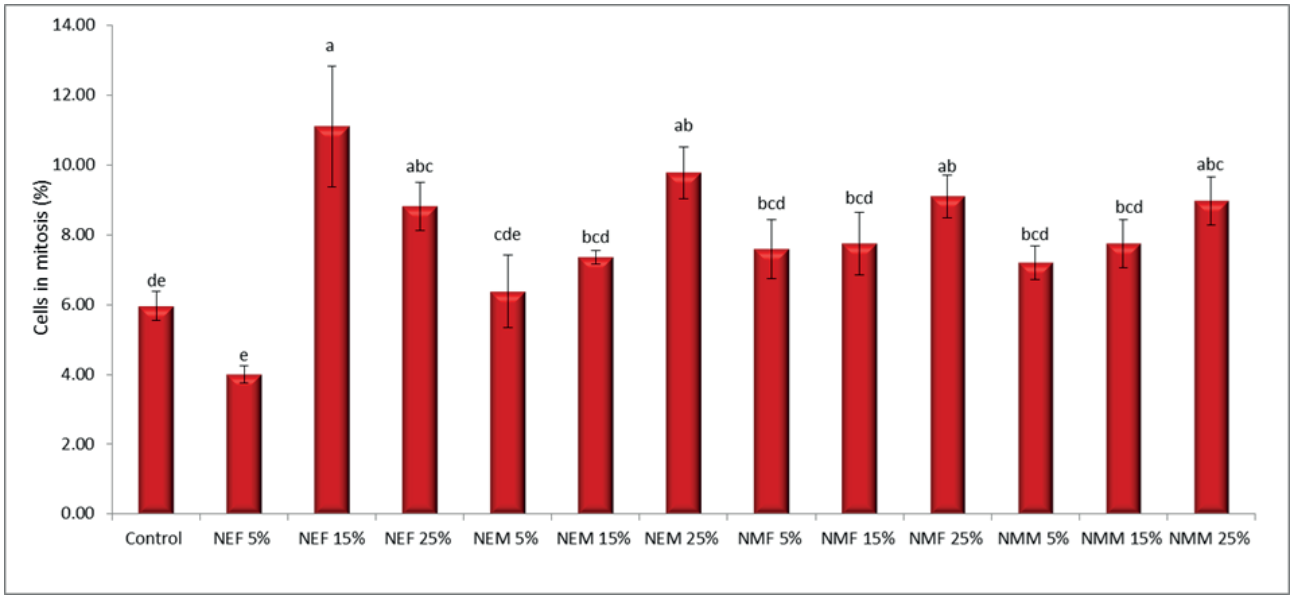

Figure 1. Influence of ethanol and methanol female and male extracts of *N. viridula* on the mitotic index in the meristematic root cells of *A. cepa* (a, b, c, d, e: interpretation of significant differences using Duncan's test, $p<0.05$).

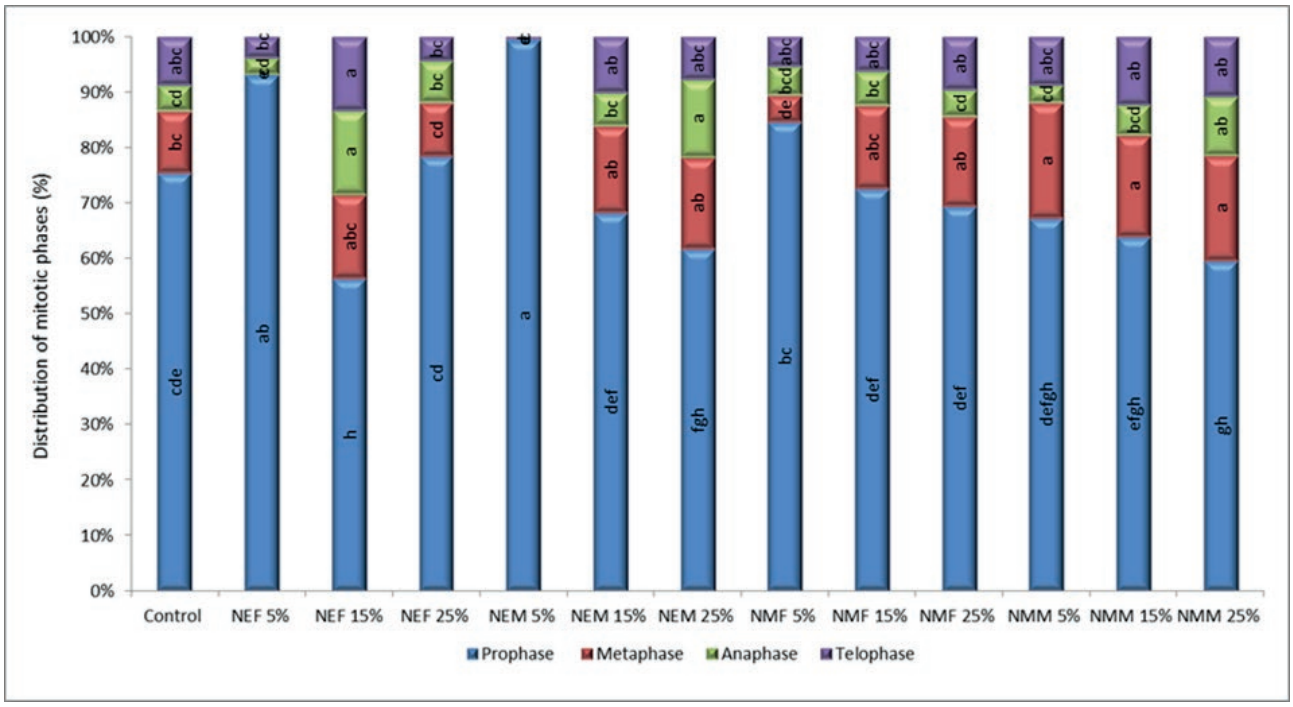

Figure 2. Influence of alcoholic extracts of *N. viridula* on the distribution of the phases of mitotic division in the root meristem cells of *A. cepa* (a, b, c, d, e, f, g, h: interpretation of significant differences, using Duncan's test, $p<0.05$).

C-mitoses, vagrant and laggard chromosomes, polyploidy, fragments of chromosomes, but also some mitotic anomalies such as micronuclei, binucleate cells or nucleoplasmic bridges (Figure 3) were observed with a variable frequency in root tip cells. The incubation of onion roots in extracts of *N. viridula* did not produce significant differences in the total frequency of chromosomal and mitotic aberrations, except for NEF 25%, the sample with the highest frequency.

Of the interphase anomalies, micronuclei were observed in the experimental samples defined by extracts at 15% concentration, and the nucleoplasmic bridges were more frequently identified in the cells treated with extracts of *N. viridula* female, irrespective of the solvent. Laggards, stickies, anaphase bridges and multipolar anaphases were predominant in the tested samples. The frequency of sticky chromosomes was ranging between 20.06% in the sample NMM 5% and 90.47% in the sample NEF 15%, and the anaphase bridges varied between 5.55% in the NMM 5% and 91.67% in NMF 15%.

Antimicrobial activity of male and female extracts of N. viridula

The antimicrobial effect of *N. viridula* extracts is presented in Table 4.

The largest zones of growth inhibition were observed for *Candida albicans* (between 11.33 mm and 15 mm) and *C. parapsilosis* (between 10.66 mm and 12.66 mm), under the action of ethanol extracts. While the same inhibition zone was induced by extracts and negative control in *C. albicans*, *C. parapsilosis* was sensitive to ethanol extracts.

The inhibitory effect produced by insect extracts on *B. subtilis* had almost the same value with the one for *C. parapsilosis*. Both ethanol and methanol extracts had a greater impact against bacteria than negative controls (an exception occurred for methanol extracts from females of *N. viridula*). With smaller values for the diameter of the inhibition zones than *B. subtilis*, but bigger than the negative controls, the *E. coli* strains demonstrated a sensitivity to insect extracts.

However, *S. aureus*, in correlation with its well-known resistance to many antimicrobial substances (Foster 2017), and *S. pyogenes*, revealed the smallest zones of growth inhibition. The *S. aureus* strain had a slight sensitivity to the methanol extract of males of *N. viridula*, and *S. pyogenes* demonstrated a slight sensitivity to the ethanol extract of males of *N. viridula*; the other values were smaller than those to negative controls.

Certain bacteria were influenced by insect extracts, depending on the gender of insects. For instance, for

Table 3. Frequency of chromosomal aberrations and mitotic anomalies induced by the action of alcoholic extracts of *N. viridula* in the root meristems of *A. cepa* (a, b, c, d, e, f, g interpretation of significant differences using Duncan's test, $p<0.05$).

Exposure variants	Micro-nucleus	Binucleate cells	Nucleoplasmic brisges	Vagrant forms	C-mitoze	Sticky chromosomes	Anphase bridges	Multipolar anaphase	Telophase bridges	Laggards forms	Others	Total
Control	-	0.2±0.03ab	-	-	-	35.74±12.16abc	14.09±5.9de	0.32±0.32b	-	0.32±0.32b	0.41±0.41a	1.26±0.41b
NEF 5%	0.65±0.21a	0.22±0.05ab	0.33±0.33b	-	-	-	-	0.27±0.27b	2.08±2.08 ab	0.69±0.12a	0.17±0.06a	2,5±0.32b
NEF 15%	-	0.45±0.45a	16.66±9.62a	-	-	90.47±4.76a	-	-	-	-	0.14±0.1a	3.32±0.76b
NEF 25%	-	-	3.45±1.03b	-	-	77.5±11.46ab	76.39±6.06abc	-	-	-	0.03±0.03a	9.48±2.39a
NEM 5%	0.96±0.22a	0.18±0.06ab	-	-	-	-	-	0.33±0.33b	-	0.33±0.33 b	0.64±0.27a	2.03±0.51b
NEM 15%	-	-	-	1.75±1.75a	-	85.67±7.69ab	77.78±14.7ab	0.06±0.06c	-	1.45±1.45 a	-	1.43±0.26b
NEM 25%	-	-	-	-	-	86.75±6.88ab	78.71±7ab	-	-	-	-	2.46±0.22b
NMF 5%	0.81±0.45a	-	0.04±0.04b	-	-	73.81±14.48ab	35.55±19.37cd	0.11±0.11c	-	0.11±0.11b	0.05±0.03a	1.57±0.35b
NMF 15%	-	0.04±0.04ab	0.1±0.1b	-	-	61.52±6.24abc	91.67±8.33a	0.96±0.53a	-	0.96±0.53 b	-	2.55±0.43b
NMF 25%	-	0.08±0.04ab	0.69±0.35b	-	-	37.61±1.81abc	41.66±12.73cd	0.19±0.09c	-	0.19±0.09b	0.03±0.03a	1.74±0.37b
NMM 5%	0.15±0.06b	0.03±0.03ab	0.18±0.14b	2.22±2.22a	68.53±11.88a	20.06±0.77bc	5.55±5.55e	0.1±0.06c	9.52±9.52 a	2.56±2.56a	4.76±4.76a	3.41±0.7b
NMM 15%	-	-	-	-	1.19±1.19b	65.28±63.65abc	61.11±13.89abc	-	-	-	-	2.21±0.48b
NMM 25%	-	-	-	-	6.95±3.56b	64.68±4.57abc	50.53±3.68bc	-	-	-	-	1.57±0.13b

Figure 3. Chromosomal and mitotic aberrations identified in the root tips of *A. cepa* exposed to the extracts of *N. viridula*: (a) sticky chromosomes – NEM 15%; (b) anaphase bridges – NEM 25%; (c) C-Mitosis – NMM 15%; (d) giant cell – NEF 25%; (e) vagrants – NEM 15%; (f) micronucleus – NMF 15%; (g) binucleate – NEM 5%; (h) polyploidy – NMM 15%; (i) telophase bridge and chromosome fragment – NEF 25%; (j) laggard – NMF 15%; (k) multipolar telophase – NMM 15%; (l) star polar anaphase – NEM25%; (m) multipolar anaphase – NMM 15%; (n) nucleoplasmic bridges – NEF 5%; (o); (p) apoptotic bodies – NMF5%.

Figure 4. The inhibition zones induced by alcoholic extracts of *Nezara viridula* (L.): (a) Gram negative bacteria - bacillus; (b) Gram positive bacteria - bacillus; (c) Gram positive bacteria - coccus; (d) Eukariotic microorganism - yeast.

B. subtilis the inhibition zones had higher diameters by the ethanol extracts from females of *N. viridula* and the methanol extracts of males of *N. viridula*. At the same

Table 4. The diameter of inhibition zones induced by alcoholic extracts of *N. viridula*.

Experimental variants	Inhibition zone (mm) *S. aureus*	Inhibition zone (mm) *S. pyogenes*	Inhibition zone (mm) *B. subtilis*	Inhibition zone (mm) *E. coli*	Inhibition zone (mm) *C. parapsilosis*	Inhibition zone (mm) *C. albicans*
NEM	8.66	9.33	12.66	11.66	12.66	15
NEF	8.66	8.66	13.33	11.66	12.33	15
NMM	9.33	7.33	11.66	9	10.66	12
NMF	7.66	7.66	9	9	11.33	11.33
Ethanol (E)	8.66	9	11.66	10	12	15
Methanol (M)	8.66	8	9	8	11.33	14
Gentamicin 10 μg (ATB)	25	28	31	25	-	-
Fluconazole 25 μg (AM)	-	-	-	-	37	25

time, *Escherichia coli* were equally inhibited by the extracts from males or females of *N. viridula*.

All the values obtained for the growth inhibition zones under the action of insect extracts were smaller than the values for positive controls (either antibiotic or antifungal agents).

DISCUSSIONS

Statistical analysis of the data revealed differences and significant differences of the cytogenotoxic endpoints under research. Aldrich et al. (1978) found that the repugnant defensive secretion from both males and females of *N. viridula* contains (*E*)-2-hexenal, hexanal, 1-hexanol, and *n*-tridecane. Although the above authors found the *n*-tridecane content was three times more than males, in our study mitosis progression was influenced by gender only by ethanolic samples which induced the highest and the lowest MI. One of the main mechanisms involved in cytotoxicity and genotoxicity is the overproduction of reactive oxygen species (ROS) that can induce reversible and irreversible changes in proteins and cause DNA damage (Zhu et al. 2013; Choudhury et al. 2016; Tanaka and Hadwiger 2017). In this context, it is worth mentioning that aldehydes, such as hexanal can act as secondary messengers of oxidative stress controlling cell proliferation, cell differentiation and cell death (Barerra et al. 2008; Barrera 2012). The cytotoxicity of extracts, which was dependent on concentration, could be attributed to epoxides (Marshall and Caldwell 1996), which are the main constituents of pheromones in the males of *N. viridula* (Brézot et al. 1994).

The cytotoxic effect of the *N. viridula* extracts was manifested through an increase in prophase frequency and a decrease in metaphase frequency, suggesting that cells underwent mitosis, but were arrested during prophase. Inhibition of an early mitotic stage could be due to the alteration of the chromosome condensation mechanism or the inhibition of the microtubule assembly mechanism, which leads to prometaphase arrest (Oliva et al. 2002). NMM, irrespective of their concentration, induced a significant metaphase arrest in root tip cells suggesting the disturbed spindle function, which produced C-mitosis and polyploidy (Figure 3).

Chromosomal aberrations and micronuclei are biomarkers of genotoxicity and chromosomal instability determined by mutagenic agents (Bonciu et al. 2018). As complex mixtures, the extracts act as clastogenic agents inducing the formation of ana-telophase bridges and micronuclei, but also as aneugenic agents by inducing delays, adherence and multipolarity (Leme and Marin-Morales 2009). However, analysing the total frequency of chromosomal and mitotic aberrations it was noticed that NEF 25% induced the highest genotoxicity. The analytical characterization of the extracts could add a clue regarding the high genotoxicity, especially for the ethanol female extracts of *N. viridula*. Until now, literature analysis provides scarce information on the cytogenotoxic effects of extracts obtained from insects, in terms of investigating their therapeutic potential. A series of studies noticed the absence of cytogenotoxic effects in the case of extracts obtained from edible insect species, including *Zonocerus variegatus*, *Oryctes boas* (Memiş et al. 2013), *Onitis* spp., *Caelifera* spp. and *Gryllotalpa* spp. (Koc et al 2014), *Locusta migratoria* (Turkez et al. 2014), confirming the safety of their consumption by humans. Several recent papers presented the results of research on the genotoxic effects of extracts obtained from other invertebrate animals. Jayathilake and Jayewardena (2021) investigated with the *Allium* test aqueous extracts from the sea cucumber, *Bohadschia vitiensis*, known for certain biological activities and found reduced genotoxic effects consisting of 0.1-0.2% chromosomal aberrations

including chromosomal bridges, c-mitosis, chromosomal breaks, and vagrants. By using the *Allium* bioassay, the mitodepressive effect of the marine sponge extracts *Luffariella herdmani* was highlighted, the results obtained suggesting the antitumor potential of the substances contained (Kuruppuarachchi et al. 2023).

In our study, the antimicrobial effect of insect extracts was noted especially for ethanol extracts of *N. viridula* against those microorganisms which usually are opportunistic pathogens.

The bacteria and yeast with higher pathogenic properties (*S. aureus*, *S. pyogenes* and *C. albicans*) are less sensitive to the action of extracts, although other studies emphasized that Methicillin-resistant *S. aureus* can be inhibited by extracts from insects used in traditional Chinese medicine (Ma et al. 2019) or that *Candida albicans* growth can be inhibited by peptides from supermeal worm, *Zophobas morio* (Fabricius) (Faruck et al. 2017).

Some studies found the antimicrobial activity of certain edible insects to their microbiota, particularly in the case of antimicrobial peptides that can be used for developing new drugs against multidrug-resistant pathogens (Mudalungu et al. 2021). Antimicrobial peptides (AMPs) from insect sources were also used to reduce biofilm associated *S. aureus* and *E. coli*; the authors found out the AMPs combined with antibiotics may be a better alternative than antibiotics alone (Sahoo et al. 2021).

The effect of ethanol as a negative control was mostly higher than methanol and the diameter of inhibition zones was the largest for positive control (antibiotic or antifungal agent). Neither bacteria nor yeasts were affected by insect extracts more than by standard antimicrobials (Figure 4). The results revealed some differences between extracts according to the gender of insects, but no obvious correspondence was established.

CONCLUSION

The mitotic index recorded after the exposure of the onion roots to the action of alcoholic extracts of *N. viridula* indicated specific and significant variations in relation to solvent and dilution. The ethanolic extracts determined the widest variation of the mitotic index, regardless of the tested concentration, while the methanolic extracts had significant mitostimulatory effects only in higher concentrations. The lower concentration extracts were associated with a blocking of cells in prophase, while the extracts obtained from *N. viridula* males determined the increase in the frequency of metaphases.

The antimicrobial effect of alcoholic extracts from *N. viridula* was obvious against bacterial strains *Escherichia coli* and *Bacillus subtilis*; *Staphylococcus aureus* and *Streptococcus pyogenes* presented only a slight sensitivity to the insect extracts. The differences between male or female insect extracts regarding their antimicrobial activity were unsteady, requiring further investigation into the production and specific application of insect extracts.

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Apogamous *Isoetes coromandelina* L.f. (Isoetaceae) with asynaptic meiosis

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Abstract. This cytological study on *Isoetes coromandelina* L.f (Isoetaceae) from Tirunelveli District, Tamil Nadu, South India shows the presence of twenty two individual chromosomes along with a fragment (n=2n=22+1) during meiosis in megaspore mother cells without the presence of any bivalent. Cytomixis between two megaspores is very common, resulting in the formation of a high percentage of abortive spores. The present material is of an apogamous taxon with asynaptic Meiosis as reported by Abraham and Ninan from Kerala and Karnataka.

Keywords: *Isoetes coromandelina*, asynaptic meiosis, apogamy.

INTRODUCTION

Lycophytes and Pteridophytes form the dominant vegetation on the earth next to flowering plants. Fern allies and ferns are a monophyletic group and the closest living relatives to seed plants (Pryer *et al*., 2001). This group of plants, with homosporous clubmosses, heterosporous spikemosses and quillworts, is the ancestor for Lycopodiopsida and Monilophytes Smith *et al*. (2006). Quillworts are considered by some to be the last remnant of the fossil tree lycopsid with which they share some unusual features including the development of wood and bark, a modified shoot system acting as roots, bipolar growth, and an upright stance (Retallack, 1997). *Isoetes,* one of the extant members of Lycopodiopsida, is a heterosporous fern. They are small aquatic herbs and they are commonly called quillworts or Merlin grass. Many aquatic plant populations including quillworts are under severe threat in the Western Ghats, particularly in Kerala and Maharashtra. For example, the endangered *Isoetes panchganiensis* has been reported from temporary ponds and pools on the high altitude plateaus of Panchgani table lands in Maharashtra and Kemmangundi Hills in Karnataka (Molur *et al*., 2011). Some aquatic species, often found in mixed populations containing taxa of different ploidy, appear to have evolved abruptly via interspecific hybridiza-

tion and chromosome doubling. Evidence from distribution patterns, megaspore morphology and viability, chromosome numbers, and electrophoretic profiles of leaf enzymes supports a hypothesis of allopolyploid speciation (Taylor & Hickey, 1992).

Isoetes is not only a very complicated genus but also a remarkable genus with lot of things yet to be studied and understood. From India, large number of species under *Isoetes* has been reported as new species. After Fraser-Jenkins *et al*. (2017) it is clear that there are only four different species (*I. coromandelina* L.f., *I. dixitii* Shende, *I. sahyadrii* Magab. ex L. N. Rao, *I. udipiensis* P. K. Shukla, G. K. Srivast., S. K. Shukla and P. K. Rajagopal) of *Isoetes* in India. As the species of *Isoetes* are mostly aquatic and Tamil Nadu can have low rainfall for several years with rare occurrence of normal rainfall, the occurrence of *Isoetes* species is rare and seasonal. But once there is enough rainfall, they flourish immediately and cover large area in several acres as mono-dominant species which is the characteristic features of several related fossil lycophytes like *Pleuromeia rossica,* which grew in wet shore side habitats and formed mono-dominant communities. Such ecological type of a mono-dominant plant community is quite typical of such hygrophilous vegetation in both the geological past and the present-day world, but it is inhabited by taxonomically different plants. The occurrence of mono-dominat *Isoetes coromandelina* L. f. has been reported from Kallidaikurichy, Tirunelveli District by Manickam and Irudayaraj (1992) and from a lake near Bharathidasan University, Tiruchirapalli, Tamil Nadu on 1999. They have mentioned that although the species is common throughout India, it is a rare species in Tamil Nadu. With the presence of rainfall above the average amount during the year 2014, this aquatic weed started to grow in and around several ponds and water pits in several localities, including the same locality Kallidaikurichy of Manickam and Irudayaraj (1992). Thanks to the availability of this seasonal aquatic weed, we aimed to make detailed cytological studies on the quillwort *Isoetes coromonandelina* L. f.

MATERIALS AND METHODS

Materials for the present study were collected from Kallidaikurichy, Tirunelveli District, Tamil Nadu, India during 2014 (Figure 1 A,B). Sporangia (Figure 1 C, D) were fixed in the mixture of Absolute alcohol, Chloroform and Acetic acid (6:3:1). Acetocarmine squash technique was followed to study the chromosomes behaviour in megaspore mother cells. Voucher has been deposited in St. Xavier's College Herbarium, Palayamkottai.

RESULTS AND DISCUSSION

Meiosis was observed in megaspore mother cells. Thin, network chromosomes, without the identity of individual chromsosme were observed in the leptotene stage and long, thick, rod like overlapping chromosomes were observed in zygotene stage (Figure 2, A-C). Pachytene and diplotene stages were not observed. After zygotene stage, each and every chromosome remains as such without pairing of homologous chromosomes. Instead of bivalents formation, they all remain as univalents. Thus all the chromosomes were like that of mitotic ones. There are 22 stout and thick chromosomes of more or less uniform size along with a very short chromosome. The chromosomes are of variable in length and type (Figure 2, D-E). The meiosis is more or less regular from I metaphase to II telophase except the irregular behavior of the extra chromosome (22 + 1) which either forms Chromosome Bridge or lies little away from others during first anaphase and telophase (Figure 2 F-I). There is no indication for the presence of this extra chromosome, in the form of micronucleus, in tetrads or young spores (Figure 2 L-Q). The mature megaspores are in different size and the surface of the megaspores is with prominent tubercles (Figure 1, E-H).

The careful observation on number of chromosomes in each group of Anaphase I, clearly shows the unreduction of chromosome number during first meiosis (Figure 2, F, G, H). From the present study on meiosis in megaspore mother cells of *Isoetes coromandelina* L.f from Kallidaikurichy, Tirunelveli District, Tamil Nadu, it is concluded that the chromosome number is n=22 +1. This is the first count for *I. coromandelina* L. f from Tamil Nadu and it is in agreement with Abraham and Ninan (1958), who have observed the presence of complete asynapsis in this species in populations from Kerala and Karnataka. They have concluded that this species from South India is of asynaptic apogamous form.

Cytological reports on Indian Quillworts are available. Plants of *I. panchananii, I. indica* and Varanasi plants of *I. coromandelina* are with forty-four chromosomes and a fragment at mitosis in root tip smears but the plants of *I. coromandelina* growing in Lohgarha and Konark are with only thirty-three chromosomes and a fragment (Pant & Srivastava, 1965). As a rule, the first meiotic division shows almost complete asynapsis, most of the chromosomes being univalents although a few are bivalents and multivalents. The association of bivalent and multivalent chromosomes takes place in various ways. About 50% of the spores are nucleate and the remaining 50% are enucleate, which again suggests that a second nuclear division is normally absent in the spore mother cells of

Figure 1. A. Large colony of *Isoetes coromandelina* L.f. in Kallidaidurichi, Tirunelveli District, Tamil Nadu, India, B. Habit, C-D. Sporophylls, E-H. Megaspores under light microscope (E, G) and Electron Microscope (F,H).

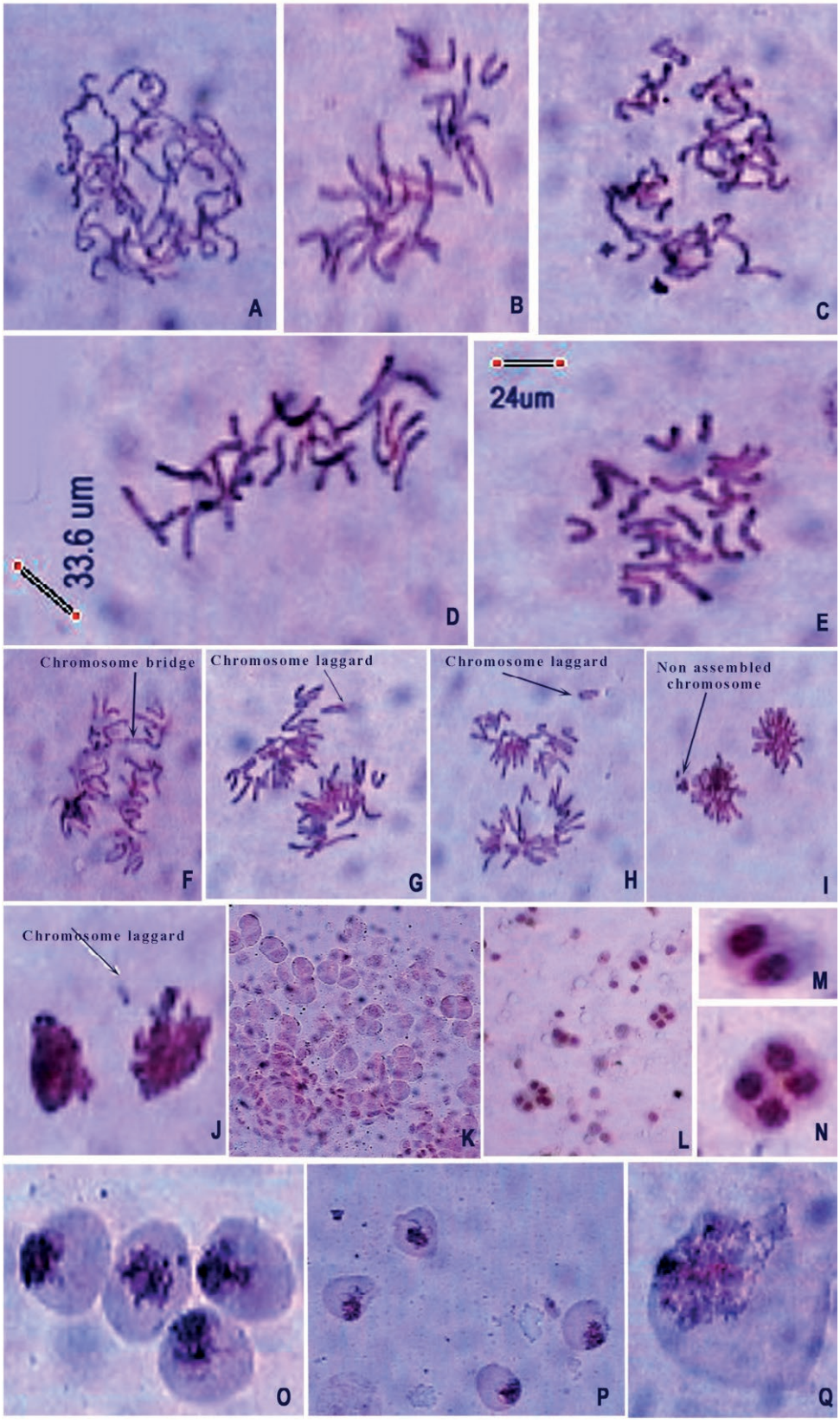

Figure 2. Meiosis in megaspore mother cells of *Isoetes coromandelina* L. f. A-J Meiosis I. K-N. Diads and tetrads; O,P,Q- Young spores.

Indian species of *Isoetes* and that their tetrads are formed by cytokinesis of the two dyads. The young larger megaspores of *I. coromandelina, I. indica* and *I. panchananii* usually show one nucleus and one "centrosomic plastid" while the smaller ones in the same species are enucleate with only one "centrosomic plastid". Karyomorphology on triploid *I. coromandelina* from South India shows Trisomy and translocations (Kuriachan and Ninan, 1974a, b). In general, *I. coromandelina* L.f. in India is a larger species complex with varied rhizome morphology, ornamentation of megaspore and cytology with the presence of diploid apomictic, triploid apomictic and triploid hybrid, tetraploid apomictic, pentaploid hybrid and hexaploid. *I. udipiensis* P.K.Shukla, G.K.Srivast., S.K.Shukla & P.K.Rajagopal is diploid sexual (Fraser-Jenkins *et al.* 2017). *I. dixitii* Shende is with 4x sexual and 3x, 6x sterile hybrids (Bir and Verma 2010).

In the present study, considerable percentage of microspores and megaspores are abortive ones with high reduction in size and irregular in shape. Another kind of abnormality in both microspores and megaspores is the presence of cytomixis. Mixing of chromatin materials of two spores through a tubular connection of the spore wall is a common phenomenon in the presently studied quillwort. The tubular connection is of different size. It is very thin and narrow, or very thick and short. Sometimes two spores are compactly and closely joined together without any tubular connection (Figure 3 A-S). Chromosomal abnormalities including cytomixis occur in meiotic process of pollen mother cells of many angiosperm species at high frequency, but similar phenomenon was rarely reported in lower vascular plants. Irregular chromosomal behaviour, especially cytomixis, is present in *Isoetes sinensis* Palmer (Heng-Chang *et al.,* 2007). It appeared in each stage of meiotic division from prophase I to telophase II. The cytoplasmic channels between microspore mother cells are narrow and stretched. The genetic material connection is maintained mainly by visible chromatin fiber. Cytomixis occurs between two adjacent microspore mother cells of same stage. A variety of other abnormal chromosomal behaviors, such as chromosomal stickiness, fragmentation, bridge, laggards, micronuclei are also present but with low frequency. Indeed, the cause of cytomixis formation and the evolutionary significance of it are still source of huge controversy. Bobak and Herich (1978) and Morisset (1978) believed it was just a pathological phenomenon by pharmaceuticals or mechanical pressure. Ghanima and Talaat (2003) stressed the impacts of extreme environmental factors. Nirmala and Rao (1996) thought cell fusion and chromatin degeneration were probably caused by both environmental and genetic factors.

Isoetes species are cytologically much variable with the presence of diploid, triploid, tetraploid and hexaploid species/cytotypes based on the base number x=11. Majority of them are sexual with few apogamous taxa. In *Isoetes* reticulate evolution by complex allopolyploid speciation has well established. So it is necessary to increase the sampling of *Isoëtes* looking for undescribed, missing species and additional intraspecific variation (Hoot *et al.,* 2004). Apogamy is not common among fern allies. In the present case of the apogamous *Isoetes coromandelina* L.f., it is peculiar in forming diplospores by avoiding normal reduction division during first meiosis in spore mother cells. Usually, the first meiosis is heterotypic division by the reduction of chromosome number and the second meiosis is homotypic division with normal mitosis. In the present case, both first and second meiosis are homotypic division without the reduction of chromosome number resulting in the formation of diplospores. Formation of diplospores with total asynapsis has been reported in several cases and the genetic control of total asynapsis in apogamous taxa has also been explained. The level and mode of diploid megaspore formation has been studied in full-sib diploid potato clones with either normal or desynaptic meiosis (Jongedijk *et al.,* 1991). The present observation of diplospory in the apogamous *I. coromandelina* L.f is similar one of pseudohomotypic division during first meiosis.

Apomixis has been repeatedly observed in fern lineages that experienced frequent reticulate evolution combined with polyploidization. The apomictic lineages showed no increase in speciation rate. Instead, all apomictic lineages appeared to be short lived despite some evidence for post origin diversification. In general, apomictic ferns are evolutionary dead ends in the long term but maintain the short-term potential to be highly successful in particular ecological conditions such as climates with strong seasonality (Liu *et al*., 2012).

There are several reports of asynaptic meiosis both in ferns and angiosperms. Manton and Sledge (1954) reported complete absence of chromosome pairing at meiosis in two separate wild collections of filmy fern *Hymenophyllum javanicum* Sprengel from Ceylon. In another filmy fern *Trichomanes insigne* v.d.B forma α is with normal meiosis, while forma β is with asynaptic meiosis (Mehra & Singh, 1957). The filmy fern, *Trichomanes proliferum* Bl. from Sarawak, is with asynaptic diakinesis with 108 univalents in spore mother cell and 32 spores in sporangia (Bell, 1960). Braithwaite (1964) described a new type of apogamy in *Asplenium aethiopicum* complex from Africa with complete asynapsis at diakinesis resulting in restitution nucleus which further mitotically divide to form 16 diplospores in each spo-

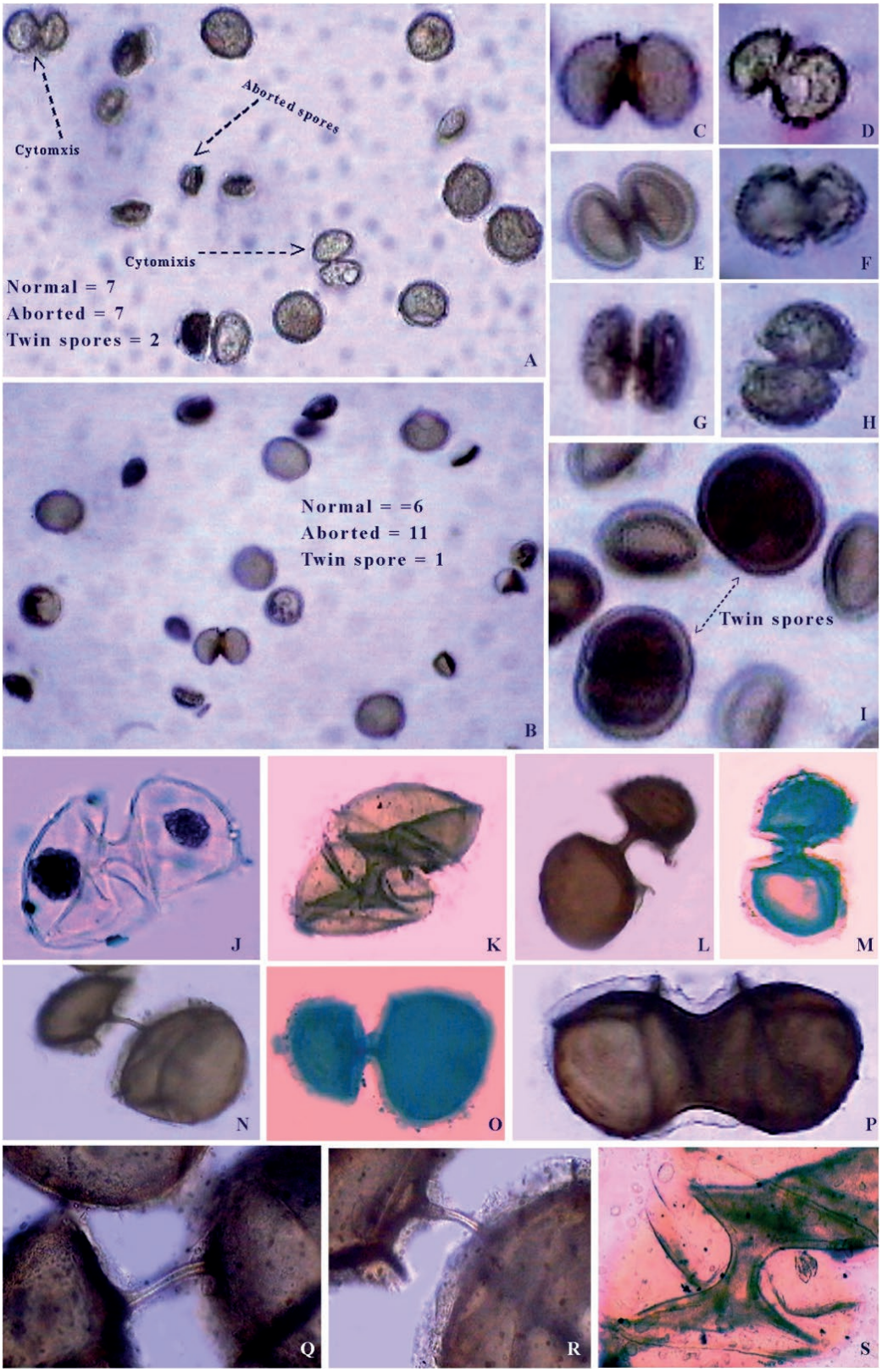

Figure 3. *Isoetes coromandelina* L.f. Cytomixis in microspores and megaspores. A,B- Mixture of spores with normal, aborted and twin spores; C-H- Cytomixis in microspores. I-Completely mingled twin microspores, J-S- Cytomixis in megaspores.

rangium. Asynaptic meiosis with the formation of unreduced gamete has been reported in interspecific hybrid of *Trifolium* (Ansari *et al.* 2022).

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Evaluation of the antigenotoxic potential of fresh bovine whey in onion meristematic roots exposed to Quizalofop-P-tefuryl

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Abstract. Whey is a protein complex derived from milk, being a functional food with multiple health benefits. In this paper, the antigenotoxic potential of fresh bovine whey (FBW) in onion (*Allium cepa*) meristematic roots exposed to Quizalofop-P-tefuryl (QPt) herbicide was evaluated using the *Allium* assay. Firstly, the *Allium cepa* meristematic roots obtained after a short germination of 24 hours in distilled water were subjected to a pre-treatment with FBW in three different concentrations (500, 1000 and 2000 µl/L) for 24 hours. After that, there was a post-treatment with QPt herbicide (100, 500 and 1000 µl/L), for another 24 hours. All variants were tested alongside a negative control (onion root tips in distilled water) and a positive control (onion root tips treated with 500 µl/L QPt). The genotoxic effects of QPt were observed in all treatment variants, through the low rate of mitosis and through the induction of a large number of chromosomal and nuclear abnormalities (bridges, laggards, rings and strap nuclei). On the other hand, the fresh bovine whey improved the mitotic activity and reduced the index of chromosomal aberrations in variable percentages, in all treatment variants. These results suggest the cytoprotective potential of FBW against the cytotoxic and genotoxic effects of the tested herbicide. Although the mechanism of antigenotoxicity is unknown, it seems plausible that the whey protein acts as a blocking agent by chemical or physical interaction with the QPt components. Nevertheless, additional studies are needed to determine with certainty this potential.

Keywords: allium assay, herbicide, genotoxic, whey, antigenotoxic.

INTRODUCTION

Genotoxicity is the ability of various agents to cause damage to genetic material and to affects cellular components involved in the functionality and behavior of chromosomes (Bhattachar 2011; Nagarathna et al. 2013). Agents able of causing genetic toxicity are described as genotoxic and are called genotoxins. One of the categories of genotoxins that cause damage to genetic material is pesticides (Anguiano-Vega et al. 2020; Kim et al. 2017).

Higher plants are used in many experiments as indicator plants that show the cytogenotoxic effects of chemicals that pollute the environment (Yıldız et al. 2009; Bonciu et al. 2018; Deveci Özkan et al. 2019). In this context, *Allium cepa* L. is a widely used indicator plant for testing the genotoxic/antigenotoxic potential of various chemicals (Bonciu et al. 2018; Datta et al. 2018; Khanna and Sharma 2013).

Pesticides (fungicides, insecticides and herbicides) are chemicals used against pests and weeds in agriculture. Certain pesticides cannot be broken down by microorganisms or the human body's enzymatic equipment, and they can accumulate. For this reason, pesticides represent a source of toxic risk, due to their persistence in soil (Datta et al. 2018; Srimurali et al. 2015), plants (Deveci Özkan et al. 2019; Roşculete et al. 2019) and in the human body (Andersson et al. 2021; Hernandez et al. 2016; Van Maele-Fabry et al. 2019; Yusa et al. 2015).

Keeping weeds under control is a constant practice of modern agriculture to ensure high yields by suppressing the growth of unwanted wild species that compete for the same resources with agricultural plants (Bartucca et al. 2019; Loddo et al. 2020). But, the frequent and excessive use of these chemicals has been identified as a serious threat to the environment and human health (Parveen et al. 2016).

Quizalofop-P-tefuryl (QPt) is a post-emergence herbicide used for the control of the grass weeds in agricultural and horticultural crops (potato, sugar beet, sunflower, oilseed rape, peanut, etc.). The active ingredient is rapidly adsorbed by the leaves of grass weeds, producing their well-wishing and consequent death. Its mode of action involves the inhibition of acetyl CoA carboxylase activity.

Whey is full of cysteine, a substance necessary for the production of glutathione, a powerful antioxidant that protects the body from infections and toxins (Gupta et al. 2017). Thus, whey helps strengthen the immune system. Glutathione is also quite effective in the treatment of the thyroid gland, cancer, sclerosis and Parkinson's disease (Marshall et al. 2004).

Current studies suggest that the fresh bovine whey (FBW) is much more than a protein source with a particularly nutritious composition of essential amino acids (Jaladat et al. 2022; Park et al. 2021; Walzem et al. 2002). Thus, FBW is a real complex cocktail, which contains, in addition to proteins, peptides, complex lipids and oligosaccharides; all of these substances act as a growth factors, toxin binding factors, antimicrobial peptides, prebiotics and immune regulatory factors in mammals (Pan et al. 2013; Teixeira et al. 2019).

Consumption of whey products can modulate redox biomarkers to reduce oxidative stress (Giblin et al. 2019). This bioactivity has been partially attributed to the whey peptides using a series of biochemical or cellular *in vitro* assays (Abdel-Wahhab et al. 2013; Corrochano et al. 2019; Garg et al. 2018; Falkowski et al. 2018).

A number of studies have demonstrated the antioxidant bioactivity of whey products and increasing glutathione levels (Brandelli et al. 2015; Yadav et al. 2015; Zhang et al. 2012). Glutathione (tripeptide with an important antioxidant role in the body of plants, animals, fungi and bacteria) prevents the destruction of some cellular components and detoxifies various endogenous and exogenous toxins (Kasperczyk et al. 2013; Pizzorno et al. 2014). Some cellular lines exposed to various whey products showed increases in glutathione levels, with some exceptions (Corrochano et al. 2019).

On the other hand, some studies suggest the adverse effects of FBW when it is consumed in excess (Amanzadeh et al. 2003; Vasconcelos et al. 2021). Practically, 20-25 grams of whey protein powder can be consumed every day, depending on the active or sedentary lifestyle. Whey is contraindicated for people allergic to dairy products and the high consumption of whey protein can lead to an increase in the percentage of body fat and stress on the kidneys, an increase in cardiovascular and osteoporosis risks, the appearance of nausea, headaches, cramps, reduced appetite, etc. (Aparicio et al. 2011; Aydın et al. 2018; Hattori et al. 2017; Vasconcelos et al. 2021).

FBW ($pH < 5.1$) as a by-product from the manufacture of hard, semi-hard or soft cheese and rennet casein is known as sweet whey. The main constituents of FBW are shown in Table 1 (Dinkci 2021).

The antigenotoxic potential of whey proteins in the field of medicine was suggested by many results (Aydın

Table 1. The main constituents of FBW.*

Constituents	%
Total solids	6.00-6.50
Water	93.00-94.00
Fat	0.05
Protein	0.60-0.65
NPN (non-protein nitrogen)	0.20
Lactose	4.50
Ash (minerals)	0.50
Calcium	0.03
Phosphorus	0.04
Sodium	0.04
Potassium	0.14
Chloride	0.09
Lactic acid	0.05

*Source: Dinkci (2021).

et al. 2018; Jaladat et al. 2022; Marshall 2004; Park et al. 2021; Teixeira et al. 2019). On the other hand, in the specialized literature there is a lack of results regarding the antigenotoxic potential of FBW following plants exposure to various chemical substances, such as pesticides. In this context, we initiated this study for evaluation of the antigenotoxic potential of FBW in onion (*A. cepa*) meristematic root tips exposed to QPt herbicide.

MATERIALS AND METHODS

Plant material

In this study, a number of ten onion (*A. cepa*, 2n=16) bulbs (procured from a local vendor) were used as biological material for each treatment variant. The outer scales were removed, and older dry roots were scrapped off in order to promote the emergence of new roots. QPt is the active substance of Pantera herbicide (producer Chemtura S.R.L. Italy). This was purchased from a local specialty store for phytopharmaceutical products and was used as the test substance. The onion bulbs were immersed in glasses with distilled water for a short germination (24 h) and then were subjected to a pre-treatment with FBW in three different concentrations (500, 1000 and 2000 µl/L) for 24 hours. After that, there was a post-treatment with QPt herbicide (100, 500 and 1000 µl/L), for another 24 hours. The QPt herbicide concentrations were established according to the dose recommended in agricultural practice. The concentrations of FBW were randomly set, because in the literature there is no data related to the testing of FBW in plants, but only some results on animals.

The length of the meristematic roots was measured and recorded after each treatment stage as roots length average (RLA). Likewise, microscopic analyses were performed after each treatment stage and for each sample, in order to determine the mitotic index (MI), the indices of mitosis phases (IP=prophase index; IM=metaphase index; IA=anaphase index; IT=telophase index) and to identify the chromosomal aberrations.

For this study, the variants were tested alongside a negative control (onion root tips in distilled water) and a positive control (onion root tips treated with 500 µl/L QPt herbicide). The experiment was performed in laboratory, at room temperature (24±2°C).

Microscopic preparations

After measuring and recording the root growth following germination, pre-treatment with FBW and respectively post-treatment with QPt, the biological material was prepared for the microscopic stage. Thus, *A. cepa* roots were fixed in ethanol: acetic acid (3:1) and hydrolysed in 1N hydrochloric acid (HCl) at 60°C for 5 min. Roots with a length of approximately 1 cm were stained through immersion in 3-5 ml Schiff reagent (30 minutes) and then transferred on clean slide and crushed in drop of 2% acetocarmine. The microscopic preparations were performed by squash technique.

All slides were labelled before microscopic analysis. Five random microscopic fields from each slide were scored.

The viewing microscopic area was divided into three viewing sections and then, in each viewing section, the cells were counted and recorded in prophase, metaphase, anaphase and telophase. The MI and mitosis phase index were calculated using the following formulas:

$$\text{MI (\%)} = \frac{\text{Total number of cells in division}}{\text{Total number of analysed cells}} \times 100$$

$$\text{IP (\%)} = \frac{\text{Total number of cells in prophase}}{\text{Total number of cells in mitosis}} \times 100$$

$$\text{IM (\%)} = \frac{\text{Total number of cells in metaphase}}{\text{Total number of cells in mitosis}} \times 100$$

$$\text{IA (\%)} = \frac{\text{Total number of cells in anaphase}}{\text{Total number of cells in mitosis}} \times 100$$

$$\text{IT (\%)} = \frac{\text{Total number of cells in telophase}}{\text{Total number of cells in mitosis}} \times 100$$

The index of the total abnormalities (ITA) was also calculated:

$$\text{ITA (\%)} = \frac{\text{Total number of aberrant cells}}{\text{Total number of cells in division}} \times 100$$

Chromosomal aberrations and nuclear anomalies were determined by scoring cells with bridges, laggards, rings and strap nucleus in randomly picked three zones per slide.

Photomicrographs of cells showing mitosis, chromosomal aberrations and nuclear anomalies were taken using the digital microscope Optika B-190TB (Optika, Italy), 1000× magnification.

Statistical analyses

The experiment was organized according to a randomized complete design with three replications and minimum 1000 cells were analysed. Statistical analysis was done using MS Excel 2016. The data obtained were analysed by one-way analysis of variance (ANOVA) and Duncan's multiple range test by using statistical software SPSS version 20 for Windows. Significance was considered at $p < 0.05$. Data were expressed as mean ± stand-

ard error (SE) (Gomez and Gomez 1984). The experiment was conducted in triplicate and minimum 1000 cells were analysed for each sample.

RESULTS

The length of the meristematic roots was measured and recorded after each treatment stage (Figure 1). Thus, after 24 hours germination in distilled water, RLA recorded values ranged between 0.3 and 0.6 cm, while the value of the negative control was 0.5 cm. After pre-treatment with FBW, the highest RLA value was found in sample V2 (1000 µl/L) - 1.9 cm, followed by V1 (500 µl/L) - 1.7 cm and V3 (2000 µl/L) - 1.6 cm. Thus, a more intense growth of onion roots is found in all variants, compared to the negative control, in variable percentages between 58.3-33.3%. On the other hand, after post-treatment with QPt herbicide, the highest RLA value was found in variant V2 (500 µl/L) - 2.3 cm, followed by V1 (100 µl/L) - 2.1 cm and V3 (1000 µl/L) - 1.7 cm. Thus, a more intense growth of onion roots is found in V2 and V1 samples, compared to the negative control, in variable percentages between 27.7-16.6%. In the case of V3 sample, the RLA value was 5.5% lower than the negative control.

Table 2 presents the results of the influence of FBW and QPt on the MI and mitosis stages index in *A. cepa* root tips.

It was found that, following pre-treatment with FBW, the MI in two variants, namely V1 (62.6%), respectively V2 (65.3%), was higher compared to the negative control (54.2%). After the treatment with 2000 µl/L FBW (V3), the MI value was lower (51.4%) than the value recorded for the negative control. On the other hand, higher values of MI were found in all variants compared to the positive control.

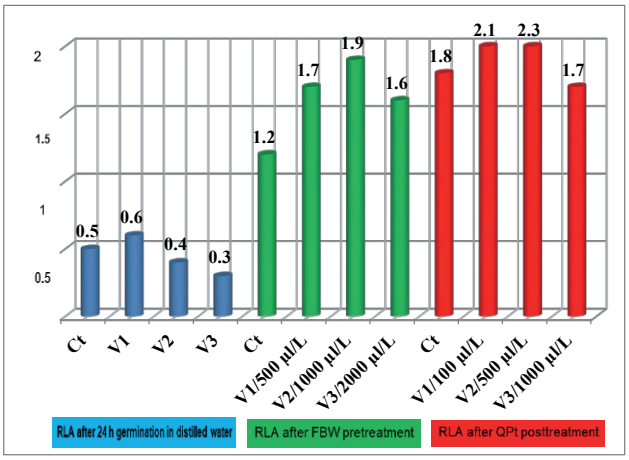

Figure 1. *A. cepa* roots lenght average (RLA) (cm) after a short germination of 24 hours in distilled water, followed by pre-treatment with FBW for 24 hours and a post-treatment with QPt herbicide for another 24 hours.

Regarding the post-treatment with the QPt herbicide, an increased MI was recorded in the same variants, namely V1 (66.2%) and V2 (71.5%), compared to the negative control. The treatment with 1000 µl/L QPt (V3) induced a decrease of MI compared to the value recorded by the negative control, but higher than the value recorded by the positive control. It can be appreciated that the 2000µl/L FBW and 1000µl/L QPt concentrations induced a mitodepressive effect in meristematic root cells of *A. cepa* in a time and concentration-dependent manner.

The indices of mitosis phases had different values compared to the control variant, between 78.7-88.4% IP, 3.0-5.5% IM, 3.1-5.1% IA and 5.5-10.7% IT, respectively.

The results regarding chromosomal and nuclear abnormalities induced by FBW and QPt in *A. cepa* root tips are presented in Table 3.

Table 2. Results of the influence of FBW and QPt on the MI and mitosis stages index in *A. cepa* meristematic roots.*

Specification	Variant/Conc. (µl/L)	MI±SE (%)	IP±SE (%)	IM±SE (%)	IA±SE (%)	IT±SE (%)
NC	-	54.2 ±1.8^{a}	82.9±0.2^{a}	3.9±0.2^{a}	5.5±0.3^{a}	7.7±0.5^{a}
PC	-	33.2 ±1.8^{b}	85.1±0.4^{a}	2.1±0.1^{a}	3.1±0.3^{a}	9.7±0.6^{a}
	V1/500/24	62.6 ± 2.1^{b}	84.2±0.5^{a}	3.5±0.4^{a}	4.2±0.5^{b}	8.1±0.5^{a}
FBW	V2/1000/24	65.3 ±2.9^{b}	83.5±0.9^{a}	3.4±0.2^{a}	3.2±0.1^{c}	9.9±0.7^{a}
	V3/2000/24	51.4 ± 3.1^{a}	85.6±0.7^{b}	3.2±0.2^{a}	4.1±0.5^{b}	7.1±0.4^{a}
	V1/100/24	66.2 ± 2.4^{b}	80.3±0.4^{a}	4.7±0.5^{b}	4.4±0.2^{b}	10.6±0.5^{b}
QPt	V2/500/24	71.5 ± 4.8^{c}	78.7±0.8^{a}	5.5±0.2^{b}	5.1±0.3^{a}	10.7±0.5^{b}
	V3/1000/24	42.7 ± 1.9^{d}	88.4±0.9^{c}	3.0±0.3^{a}	3.1±0.1^{c}	5.5±0.2^{c}

*Means with the same letter in the same column for each application time do not differ statistically at the level of 0.05. NC=negative control; PC=positive control; MI=mitotic index; SE=standard error; IP=prophase index; IM=metaphase index; IA=anaphase index; IT=telophase index. Data are means ± SE of three replicates.

Table 3. Results regarding the chromosomal and nuclear abnormalities induced by FBW and QPt in *A. cepa* root tips.*

Specification	Variant/ Concentration (µl/L)/Exposure time (h)	B (%)	L (%)	R (%)	SN (%)	ITA±SE (%)
NC	-	0.2	0.8	0.1	1.2	2.3±0.5[a]
PC	-	4.8	6.2	2.7	7.4	21.1±1.1[b]
FBW	V1/500/24	2.0	4.2	1.5	2.2	9.9±0.4[a]
	V2/1000/24	2.7	4.5	1.9	3.1	12.2±0.7[a]
	V3/2000/24	3.4	5.1	2.2	3.9	14.6±0.6[a]
QPt	V1/100/24	2.4	4.0	1.9	3.0	11.3±0.4[a]
	V2/500/24	3.5	4.1	2.2	4.3	14.1±0.4[b]
	V3/1000/24	4.1	5.4	2.1	5.3	16.9±0.9[c]

*Means with the same letter in the same column for each application time do not differ statistically at the level of 0.05. NC=negative control; PC=positive control; B=bridges; L=laggards; R=rings; SN=strap nucleus; ITA= index of the total abnormalities; SE=standard error.

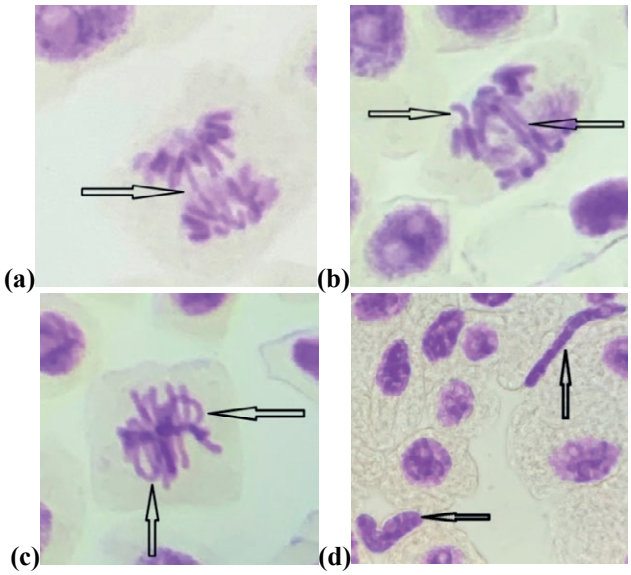

Figure 2. The main chromosomal aberrations and nuclear anomalies induced by pre-treatment with FBW and post-treatment with QPt in *A. cepa* cells: bridge (a), double bridge and laggard chromosome (b), ring chromosomes in metaphase (c) and strap nuclei (d). Arrows indicate abnormalities.

In all treatment variants, the appearance of a variable number of chromosomal aberrations and nuclear anomalies was observed, the most important being the following: bridges, laggards, rings and strap nuclei (Figure 2). Thus, the bridges were observed with a frequency ranged between 2.0-3.4% after the treatment with FBW and an increased frequency of 2.4-4.1% after the treatment with QPt, respectively. Laggards had values between 4.2-5.1% after the treatment with FBW and respectively 4.0-5.4% in the case of treatment with QPt. Also, cells with ring chromosomes had values between 1.5-2.2% in the case of treatment with FBW and respectively 1.9-2.2% in the case of treatment with QPt. On the other hand, the nuclear abnormalities of the strap nucleus type were recorded with a frequency of 2.2-3.9% and 3.0-5.3%, after the treatment with FBW and QPt, respectively. It was found that all these abnormalities recorded higher values than the negative control but much lower than the positive control. A similar evolution of the records was determined for the index of the total abnormalities (ITA%), that ranged between 9.9-14.6% (after FBW treatment) and 11.3-16.9% (after QPt treatment).

DISCUSSION

In the last years, many researchers have found novel bioactive phytocompounds able to counteract the effects of some physical and chemical mutagens that can affect the health of humans, animals and the environment (Bonciu et al. 2018; Dimitrov et al. 2006; Franco-Ramos et al. 2020). The ability of a substance to cause cytotoxicity is measured by its capability to decrease cell proliferation (Franco-Ramos 2020).

The challenge of identifying and developing of some therapies through plants help or some dietary strategies represents a major public health challenge. Thus, several studies have shown the antigenotoxic potential of different plants (López-Romero et al. 2018; Park et al. 2018; Stavric et al. 1996). Also, whey protein supplementation is a dietary strategy widely used in the field of oncology (Abdel-Wahhab et al. 2013; Gupta and Prakash 2017; Teixeira et al. 2019). This emerging dietary strategy harbouring several benefits translated well into animal models of cancer and in humans. At the molecular level, whey protein subfractions display appealing anti-cancer effects (Teixeira et al. 2019).

In order to highlight the cytological activity and the occurrence of some cytogenetic abnormalities induced to QPt in plant meristematic roots it was chosen *Allium* test because is one of the simplest, inexpensive and valuable tests for determining the cytotoxicity of chemical substances on plants. As Shetty et al. (2017) states, *A. cepa* root chromosomal aberration assay was chosen as it offers an easily adaptable method for screening mitotic/genotoxic/antigenotoxic activity of any bioactive chemical.

In our study, in all QPt herbicide treatment variants, the appearance of a variable number of chromosomal aberrations and nuclear anomalies was observed, the most

important being bridges, laggards, rings and strap nuclei. The results obtained showed the strong cytotoxic effect of QPt herbicide, by reducing the MI in onion roots. Thus, it can be appreciated that the 1000 µl/L QPt induced a mitodepressive effect in meristematic root cells of *A. cepa* in a time and concentration-dependent manner.

In this context, there are many results regarding the cytogenotoxic effects of herbicides on plants or animals. Thus, the results obtained by Dimitrov et al. (2006) show that the tested herbicide (Stomp) did not induce chromosomal aberrations in plant cells of *Crepis capillaris* L., instead it was increased their incidence in mouse bone marrow cells. In the same study on the other hand, the herbicide increased the frequency of micronuclei in both test systems. The authors suggest that the induction of some nuclear and chromosomal aberrations in plant cells may have been due to the herbicide's disruptive effect on the spindle, as all herbicide concentrations produced C-mitosis. Also, the increased frequency of chromosomal aberrations in mouse bone marrow cells may be due to the biosynthesis of genotoxic metabolites (Dimitrov et al. 2006).

Some dairy products (milk and yogurt) supplemented with red ginseng extract have been shown to have antioxidant and antigenotoxic effects (Park et al. 2018). Also, some studies have shown the potential of lactobacilli and bifidobacteria in dairy products to inhibit the genotoxic activity of chemical compounds (Burns et al. 2000; Lopitz-Otsoa et al. 2006; Tavan et al. 2002).

Our results showed that FBW has improved mitotic activity and reduced chromosomal abnormalities in the meristematic cells of *A. cepa* induced by QPt, a fact that suggests the antigenotoxic potential of FBW. The FBW reduced the aneugenic and clastogenic effects of QPt in *A. cepa* cells. Although the mechanism of antigenotoxicity is unknown, it seems plausible that the whey protein acts as a blocking agent by chemical or physical interaction with the QPt components. However, the continuation of studies in this direction remains open in order to establish with certainty the antigenotoxic potential of FBW in plant cells.

CONCLUSIONS

The experimental results from the present study show that the application of QPt herbicide produced some cytotoxic and genotoxic effects on the meristematic cells of *A. cepa*. These toxic effects increased significantly at dose dependently, but application of FBW ameliorated these negative effects which arise under herbicide stress. Thus, FBW has improved mitotic activity and reduced chromosomal abnormalities in the meristematic cells of *A. cepa* induced by QPt herbicide, a fact that suggests the antigenotoxic potential of FBW. We can conclude that FBW can be able to ameliorate the abnormalities caused in *A. cepa* cells due to herbicides stress. Also, our results suggested that, although the mechanism of antigenotoxicity is unknown, it seems plausible that the whey protein acts as a blocking agent by chemical or physical interaction with the QPt components. Nevertheless, additional studies are needed to determine with certainty this potential.

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