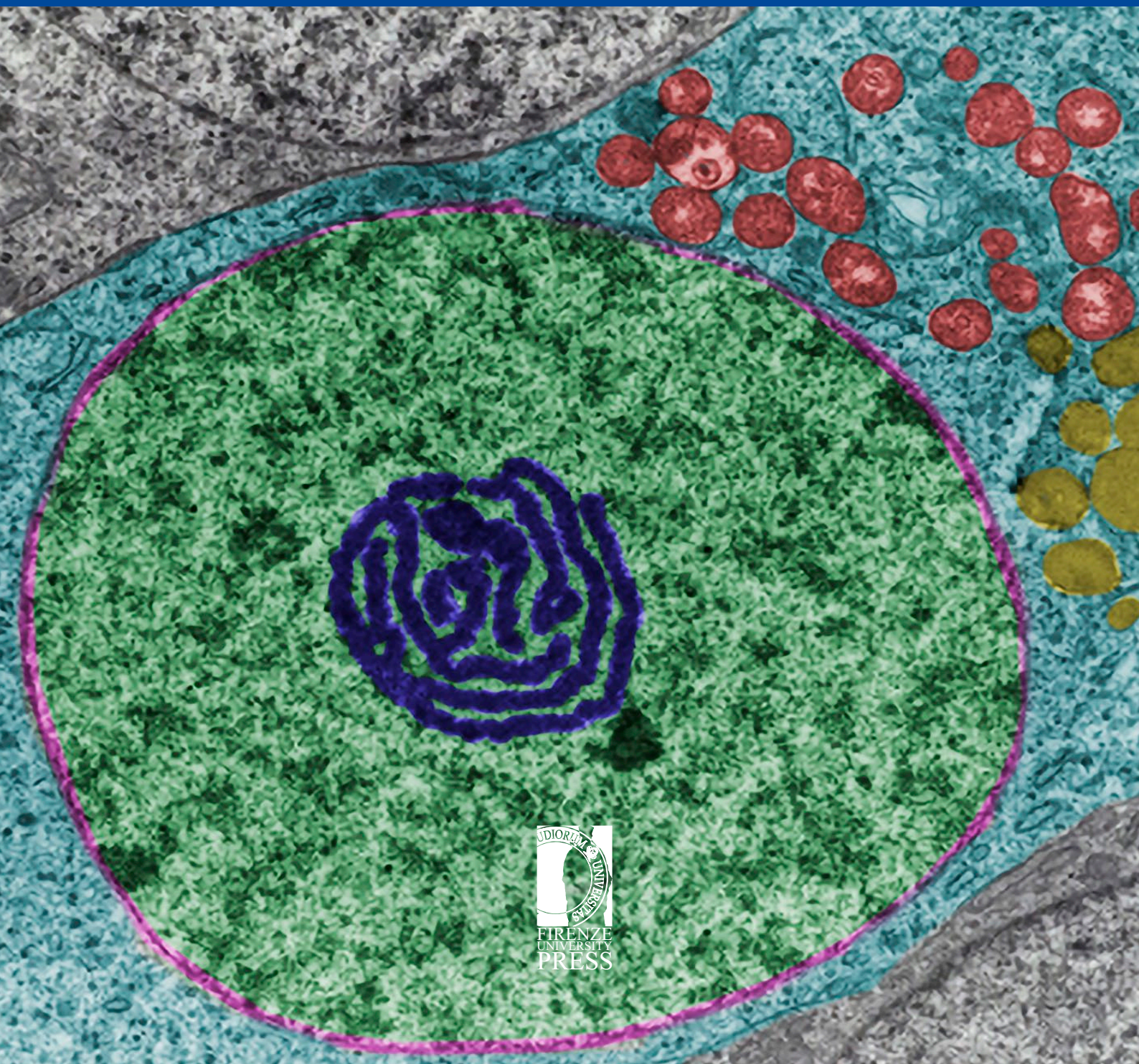
0008-7114

Caryologia

International Journal of Cytology, Cytosystematics and Cytogenetics

2019
Vol. 72 - n. 3



FIRENZE UNIVERSITY PRESS

Caryologia. International Journal of Cytology, Cytosystematics and Cytogenetics
Caryologia is devoted to the publication of original papers, and occasionally of reviews, about plant, animal and human karyological, cytological, cytogenetic, embryological and ultrastructural studies. Articles about the structure, the organization and the biological events relating to DNA and chromatin organization in eukaryotic cells are considered. *Caryologia* has a strong tradition in plant and animal cytosystematics and in cytotoxicology. Bioinformatics articles may be considered, but only if they have an emphasis on the relationship between the nucleus and cytoplasm and/or the structural organization of the eukaryotic cell.

Caryologia

International Journal of Cytology, Cytosystematics and Cytogenetics

Volume 72, Issue 3 - 2019

Firenze University Press

***Caryologia*. International Journal of Cytology, Cytosystematics and Cytogenetics**

Published by
Firenze University Press – University of Florence, Italy
Via Cittadella, 7 - 50144 Florence - Italy
http://www.fupress.com/caryologia

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: S. Tabur, M.D. Yurtlu, S. Özmen (2019) Role of humic acid against salt-induced cytotoxicity in *Hordeum vulgare* L.. *Caryologia* 72(3): 3-10. doi: 10.13128/cayologia-317

Published: December 13, 2019

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

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

Role of humic acid against salt-induced cytotoxicity in *Hordeum vulgare* L.

Selma Tabur*, Merve Dündar Yurtlu, Serkan Özmen

Süleyman Demirel University, Arts and Sciences Faculty, Department of Biology, 32260 Isparta, Turkey
*Corresponding authors: taburs@gmail.com

Abstract. The effect of humic acid, which is an replace by a biostimulant on mitotic activity and chromosome behaviors in meristem cells of *Hordeum vulgare* L. germinated under different salt concentrations were investigated. In the parallel to increasing salt concentrations, mitotic index partly decreased and observed the higher number of chromosomal abnormalities as compared to control. Also, it was determined that the mitotic index of seeds pretreated with only humic acid increased by 30% according to control and by 42% of mitotic aberrations. Whereas, humic acid along with salt significantly inhibited to mitotic index with parallel to increasing salt concentrations. Moreover, the frequency of chromosomal aberrations in seeds germinated in humic acid and salty medium significantly decreased according to its own control. Humic acid revealed to a successful performance in ameliorating of the detrimental effect of salinity in the all concentrations studied. Humic acid application at 0.35 M salinity displayed perfectly successful by reaching to the same abnormality percentage of control.

Keywords. Barley, chromosomal aberrations, cytotoxicity, humic acid, mitotic activity, salinity.

INTRODUCTION

It is a known fact to affect of abiotic stresses on plant growth and development. One of the most common environmental stress factors is salinity, which an increasing problem of many arid and semiarid areas of the World. Approximately 20% of the world's cultivated land and accounts for over 6% of the world total area is threatened by salinity (FAO 2015). Abiotic stresses, such as drought, salinity, extreme temperatures, chemical toxicity and oxidative stress are serious threats to agriculture and the natural status of the environment. Increased salinization of all arable land is expected to have devastating global effects, resulting in 30% land loss within the next 25 years, and up to 50% by the year 2050 (Wang et al. 2003).

It has been known for a long time that salt adversely affects plant growth and development, hindering seed germination, seedling growth (Çavuşoğlu and Ergin 2015), enzyme activity (Dash and Panda 2001), DNA, RNA and protein synthesis (Anuradha and Rao 2001) and mitosis (Tabur and Demir 2010 a, b; Çavuşoğlu et al. 2016). However, recent investigations are focus-

ing more on the mechanisms of salt tolerance in plants (Munns and Tester 2008).The most efficient way to minimize the detrimental effects of salinity on plant breeding is the development of varieties with high salinity tolerance. Hence, researchers have used various plant growth regulators and leaf extracts to reduce or eradicate negative effects of salinity on seed germination, seedling growth (Çavuşoğlu and Ergin 2015), and mitotic activity (Tabur and Demir 2010 a, b; Çavuşoğlu et al. 2016). However, in spite of substantial efforts, the outcome is still disappointingly poor due to the physiological and genetic complexity of this trait, the lack of reliable screening tools, and most importantly, the lack of a comprehensive understanding of the mechanisms behind salinity tolerance (Zhu et al. 2016). The data relating to mitotic activity are also mostly paradox.

The main ingredient of organic substances in the soil is humus. The most active biochemical substance of humus is humic acid. Humic substances have been known that the germination and growth of plants has stimulated. Humic substances can pass through micropores of biological or artificial membrane systems, facilitate the transport of trace elements in plant roots and behave like growth hormones in plants (Chen et al. 2004). Therefore, humic substances are evaluated as a biostimulant by du Jardin (2012) who conducted a bibliographic analysis of plant biostimulants. Biostimulants are derived from natural or biological sources and can i) enhance plant growth and development when applied in small quantities; ii) help improve the efficiency of plant nutrients, as measured by either improved nutrient uptake or reduced nutrient losses to the environment, or both; or act as soil amendments to help improve soil structure, function, or performance and thus enhance plant response (du Jardin 2012).

Humic acid has positive effects on plant growth and nutrition (Calvo et al. 2014). In cytophotometric studies of the DNA, it was seen that humic substances increased the amount of DNA synthesis in the interphase nucleus of the meristematic cells in plants (Gorova et al. 2005). Furthermore, humic substances are accepted to be a plant growth promoter, particularly by changing the root structure and growth dynamics (Jindo et al. 2012; Canellas and Olivares 2014).

Under both normal and salt conditions, there have been many investigations related to seed germination, seedling growth (Çavuşoğlu and Ergin 2015), root development (Sivananthi and Paul 2014), plant growth and mineral nutrient uptake (Khaled and Fawy 2011), and also some metabolic changes (El-Bassiouny et al. 2014). However, there is only limited research on the effect of humic acid on cell divison (Feretti et al. 2012) and the protective role of against effects of mutagenic and genotoxic of various environmental conditions and chemicals (Gichner et al. 1990; Ferrara et al. 2000). In particular, no data have been recorded about effects of humic acid on mitotic activity and chromosomal aberrations in salinity conditions.

In the study reported here, the influence of humic acid pretreatment on mitotic activity and chromosome behaviors in root meristems of barley seeds exposed to salinity stress were investigated. So, we have aimed to clarify to some extent to what extent humic acid can alleviate salt stress, whether it stimulates cells to enter the mitosis division or not and also whether it causes any changes in the structure and behavior of chromosomes or not.

MATERIAL AND METHODS

In the present study, barley seeds (*Hordeum vulgare* L. cv. Bülbül 89) were used. The barley seeds were subjected to surface sterilization before used. For this, seeds were kept in 1% sodium hypochlorite for ten minutes, then washed with distilled water five times and dried on filter paper at room temperature.

Preparation of solutions and germination of seeds

NaCl and humic acid used in the experiments were obtained from Merck and Sigma-Aldrich firm respectively. As test solution, 28 mg/L humic acid were used due to promote germination in the best way against the inhibitory effect of salt. Concentrations of NaCl were 0.25, 0.30 and 0.35 M (molar). These salt levels and the concentration of humic acid used were determined in the result of a preliminary study. Primarily, plump-looking, robust and approximately equal-sized 20-25 barley seeds were selected. Then, sterilized seeds were soaked in test tubes filled with 28 mg/L humic acid and distilled water (control) at constant volume (50 ml) for 24 h at room temperature. At the end of this pretreatment session, the seeds from every application were arranged in 10 cm Petri dishes covered with two sheets of filter paper moistened with 7 ml of distilled water and different salt concentrations. Petri dishes were transferred in an incubator to germinate at 20 ± 1°C in continuous dark for several days.

Cytogenetical analyses

When the root tips were 0.5–1 cm long, they were cut off, pretreated with a saturated solution of paradi-

chlorobenzene for 4 h at 20°C, fixed with Carnoy's Fluid I (absolute ethanol: glacial acetic acid, 3:1, v/v) for 24 h, and stored in 70% alcohol at 4°C until used. Then, the root tips were hydrolyzed in 1 N HCl at 60°C for 18 min, stained with Feulgen for 1 h, and squashed in 45% acetic acid (Sharma and Gupta 1982). After 24 h, microscopic slides were made permanent by mounting in balsam. The mitotic phases and mitotic aberrations were photographed (100×) with a digital camera (Olympus C-5060) mounted on an Olympus CX41 microscope.

Data analyses and statistical evaluations

To determine the effect of humic acid and salt on the mitotic index, at least 3000 cells (approx. 1000 per slide) were scored in control and in treated groups. Chromosomal aberrations were calculated for each concentration as the percentage of 300 dividing cells counted. Statistical analysis related to all parameters was performed by using SPSS program according to Duncan's multiple range test at the level of significance $p \leq 0.05$ (Duncan 1955).

RESULTS

Effects of humic acid on mitotic index and chromosome aberrations in normal conditions

Barley seeds pretreated with 28 mg/L humic acid were germinated at 20°C in distilled water and slides were prepared with the root tips obtained. The mitotic index values calculated as a result of the cell counting procedures performed in these slides were presented in Table 1. Humic acid pretreatment caused a 30% increase in the mitotic index of barley seeds germinated in non-stress conditions as compared to those of the control group. In other words, the mitotic index of seeds treated with humic acid was even higher than seeds germinated in distilled water.

As a result of cytological analyzes, chromosomal aberrations in meristem cells of barley seeds germinated at 20°C in distilled water (control) were statistically insignificant. That is, all mitotic phases were normal (Fig. 1). However, the frequency of chromosomal aberrations in seeds germinated in distilled water after humic acid pretreatment was remarkably higher than that in the control (Table 2). For example, while the rate of chromosomal aberrations in control seeds was 0.04%, it was 0.42% in seeds pretreatmented with humic acid.

A resulting of the application of humic acid alone, chromosomal aberrations such as fragment formation, lagging chromosome, anaphase and telophase bridges, fault polarization in telophase and anaphase were frequently observed (Fig. 2).

Table 1. Mitotic index of barley seeds germinated in distilled water and different NaCl concentrations after humic acid pretreatment.

Mitotic index (%)		
NaCl (M, molar)	Control	Humic acid (28 mg/L)
0.0 (distilled water)	*0.19 ± 0.13^{bcd}	0.27 ± 0.04^{e}
0.25	0.19 ± 0.03^{bcd}	0.17 ± 0.03^{abcd}
0.30	0.18 ± 0.01^{abc}	0.18 ± 0.02^{bcd}
0.35	0.18 ± 0.03^{abc}	0.12 ± 0.02^{ab}

The pretreatment process of seeds was performed by soaking 24 h in constant volumes of distilled water (control) or humic acid. As test solution, 28 mg/L humic acid was used. Different salt concentrations (0.25, 0.30, 0.35 M NaCl) were exogenously applied to germination medium. Data are the means of three replications ± standard deviation.

* Shows values with insignificant difference ($p \leq 0.05$) for each column shown with same letters

Effects of humic acid on mitotic index and chromosome aberrations in salinity conditions

Mitotic index scores obtained from this study made to determine the activity degree of humic acid on the

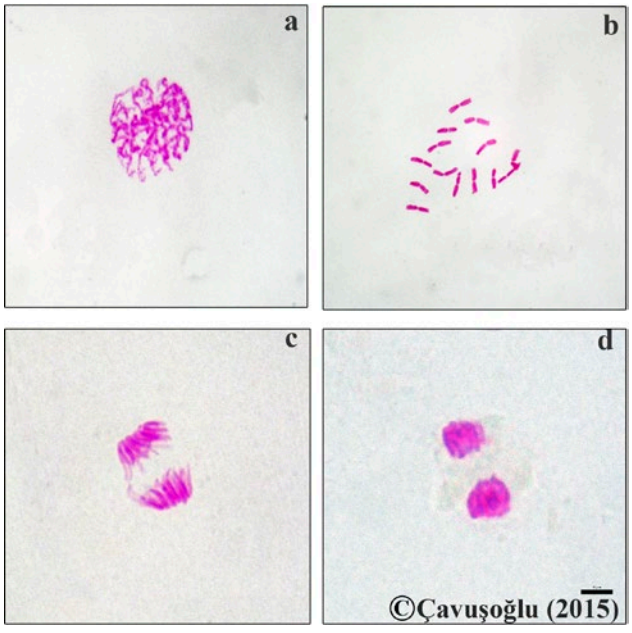


Fig. 1. Normal mitosis phases in root tips meristems of barley germinated in distilled water (control). (a) Prophase; (b) metaphase ($2n = 14$); (c) anaphase; (d) telophase. Scale bar = 10μm.

Table 2. Frequency of chromosomal aberrations of barley seeds germinated in distilled water and different NaCl concentrations after humic acid pretreatment

Chromosomal aberrations (%)		
NaCl (M, molar)	Control	Humic acid (28 mg/L)
0.0 (distilled water)	0.04 ± 0.18^{a}	0.42 ± 0.05^{cd}
0.25	0.25 ± 0.27^{bc}	0.22 ± 0.01^{bc}
0.30	0.35 ± 0.07^{c}	0.30 ± 0.15^{bc}
0.35	0.58 ± 0.05^{d}	0.04 ± 0.04^{a}

The pretreatment process of seeds was performed by soaking 24 h in constant volumes of distilled water (control) or humic acid. As test solution, 28 mg/L humic acid was used. Different salt concentrations (0.25, 0.30, 0.35 M NaCl) were exogenously applied to germination medium. Data are the means of three replications ± standard deviation.

* Shows values with insignificant difference ($p \leq 0.05$) for each column shown with same letters

mitotic index of barley seeds germinated under salt stress was summarized in Table 1.

The mitotic index value of barley seeds was statistically decreased at especially high concentrations as parallel to the increase of salt concentration as compared with control. It was found that the seeds applied alone humic acid shows a considerable increase on the mitotic index as compared with seeds germinated in distilled water. That is, the addition of 28 mg/L humic acid increased the mitotic index by 30%. However, humic acid pretreatment caused a significant decrease in the mitotic index with increasing salt concentrations. The mitotic index value (0.12) in root meristems of the seeds germinated at the highest salt concentration (0.35 M) after treated with humic acid reduced to a large extent (Table 1).

Considering all of the application groups, it was determined that humic acid pretreatment together with salinity decreased the mitotic index at 0.25 M and 0.35 M salinity, while it was at the same level with its own control at 0.30 M salinity (Table 1).

In addition to, chromosomal aberration scores were presented in Table 2. While there is a chromosomal aberration that can be ignored statistically in control seeds germinated in distilled water, screening mitotic divisions revealed the many numbers of chromosomal aberrations as parallel to increasing salt concentrations.

It was determined that chromosomal aberrations at the highest salt concentration (0.35 M) were higher approximately 60% according to those in distilled water. At the same time, alone humic acid pretreatment caused a 42% increase in chromosomal aberrations as compared to control. However, the frequency of chromosomal aberrations of seeds germinated at different salt concentrations after treatment with humic acid showed a decrease which can be considered statistically significant from those of the seeds germinated in distilled water after treatment with humic acid. Namely, humic acid has been quite successful in mitigating the detrimental effect of salt stress on chromosomal aberrations at all the salt concentrations studied here. Moreover, humic acid application at the highest salt concentration (0.35 M) significantly reduced the detrimental effect of salt stress and reached the percentage of the same abnormality (0.04) as the seeds germinated in distilled water (Table 2).

The most prominent chromosomal aberrations in seeds belonging to all application groups were the disorganizations in anaphase and telophase such as bridges,

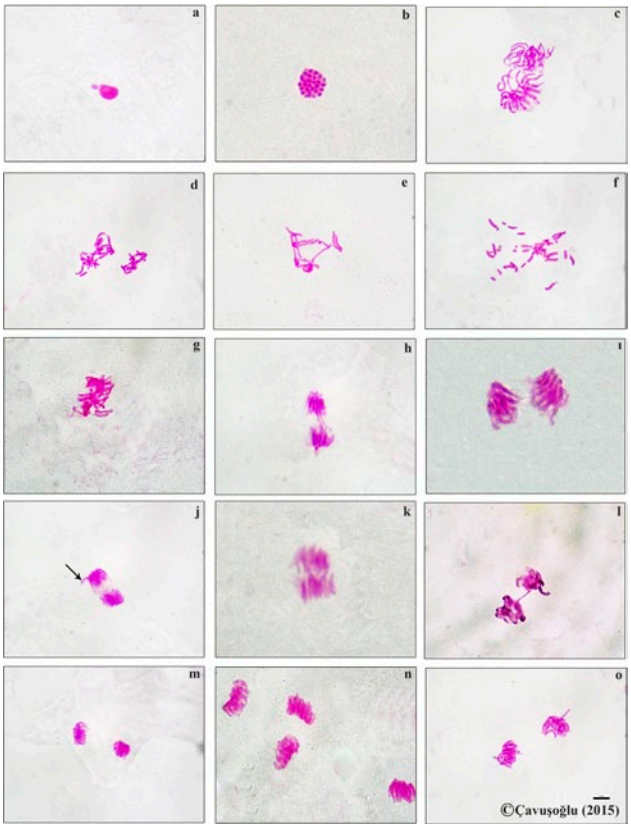


Fig. 2. Chromosomal aberrations in root meristems of barley seeds germinated in distilled water and different NaCl concentrations after treatment with 28 mg/L humic acid: a) micronucleus; b) granulation c) disorderly prophase; d) disrupted equatorial plate; e) uncoiling chromosomes f) fragments g) sticky chromosomes; h) bridges in anaphase and lagging chromosome; ı) fault polarization in anaphase; j) vagrant chromosome in anaphase (arrow); k) alignment anaphase; l) bridges in telophase; m) fault polarization in telophase n) distant poles in telophase; o) vagrant chromosome in telophase. Scale bar = 10 µm.

lagging chromosome, fault polarization, distant poles, vagrant chromosomes and alignment anaphase (Fig. 2h–o). In addition, other chromosomal aberrations observed were the presence of micronucleus, granulation, disorderly prophase, disrupted equatorial plate, uncoiling chromosomes, fragments, sticky chromosomes (Fig. 2a–g). The minimal common aberrations were micronucleus, granulation and disrupted equatorial plate.

DISCUSSION

It is well known that humic substances stimulate germination in seeds of various species by increasing enzymatic activities in seed tissues during germination and decreasing mitotic activity under normal conditions (Feretti et al. 2012). However, no information has been found on effects of humic acid on mitotic activity and chromosomal aberrations especially under saline conditions. All the data on the effect of humic acid on these parameters in saline conditions are presented for the first time in this study.

In the present work, it was determined that humic acid pretreatment alone in non-stress conditions significantly increase the mitotic index of barley seeds as compared to distilled water (Table 1). Whereas, Feretti et al. (2012) have suggested in their study using various plant tests (*Allium cepa* test, *Tradescantia* and *Vicia faba* micronucleus test) that alone humic acid pretreatment decreases the mitotic index in studied two solutions. Because of the differences in the findings of these researchers may be due to the species of plant studied or the concentration of the humic acid used. However, our findings have been endorsed by the data expressed that the humic substances increase the amount of DNA synthesis in meristematic cells in plants (Gorova et al. 2005). In addition, we found that the application of alone humic acid increased the chromosome abnormality rate by 42% under normal conditions (Table 2). Our this finding is consistent with Ferretti et al. (2012)'s findings. For this reason, we can reach the result that alone humic acid pretreatment increases the mitotic index value in barley seeds germinated in distilled water but may have a genotoxic effect because of the negativity that it has shown on chromosome behavior. This reveals the fact that alone humic acid application can create mutations in various types over time.

As is known, plant growth and development are adversely affected by salinity. High salinity is an important factor negatively influencing plant growth and development even in most halophytes. At present, approximately 20% of cultivated lands in the World are affected by salinity (FAO 2015). Generally, it is suggested that salinity impairs seed germination, retards plant development and reduces productivity. In some cases, the plant dies before completing the life cycle. There have been numerous investigations conducted to explore the effects of salt on plant growth, but mechanisms of salt stress have not yet been explained precisely (Munns and Tester 2008; Zhu et al. 2016).

We determined that mitotic index in root meristems of barley seeds significantly decreased with increasing salt concentrations (Table 1). The inhibitory effects of salt stress on mitotic activity are known for a long time (Lutsenko et al. 2005). Salt induced-inhibition of cell division may relate to osmotic effect and ion uptake (Munns and Tester 2008), inhibition of DNA, RNA and protein synthesis (Anuradha and Rao 2001), distruption the activity of enzymes required for cells (Miller et al. 2010) and hinderance of mitosis division (Tabur and Demir 2010 a, b; Çavuşoğlu et al. 2016). It is worth mentioning again that the relation between salinity and mitotic activity was confirmed by the present work. In our study, it was also detected that there was a remarkable increase in all kinds of chromosomal aberrations at the root meristems of barley parallel to the rise of salt concentrations (Table 2). The detrimental effects of salt stress on chromosomal aberrations in plants have been studied for over the past decade. These recent studies have shown that the higher concentrations of NaCl has chromotoxic effects and increases the percentage of total aberrations (Tabur and Demir 2010 a, b). Furthermore, it was reported that these high salt concentrations delayed mitosis and caused various anaphase aberrations in barley (Tajbakhsh et al. 2006) and in onion (Çavuşoğlu et al. 2016).

There is no relevant literature data relating to effects of humic acid on either mitotic activity or/and chromosomal abnormalities in saline conditions. The present study is the first one revealing the cytogenetic responses to the salt stress of humic acid. However, there are a few studies about the effect of humic acid application against the genotoxic effects of various chemicals such as N-nitrous compounds, maleic hydrolase and some disinfectants (Gichner et al. 1990; Ferrara et al. 2000). These studies have argumented that humic acid exhibits an anticlastogenic or antimutagenic activity in different plants. Ferretti et al. (2012) determined that humic acid alone reduces the mitotic index and has genotoxic effects. However, there could not be made explanation for the effect of humic acid on these disinfectants since these investigators have not determined any evidence of the genotoxic effect of disinfectants alone.

In the present work, we analyzed that humic acid pretreatment in salt stress conditions was not sufficiently successful on the mitotic index of barley seeds, but exhibit a performance very successfully statistically on chromosome behaviors. Although humic acid application alone was caused a significant increase (42%) of chromosomal aberrations in root meristems of barley seeds germinated in distilled water, it has shown remarkable success in alleviating a large majority of these abnormalities caused by salinity in salt stress conditions. In parallel to salt concentrations rise, humic acid was reduced the detrimental effects of salinity and caused complete elimination of chromosome abnormalities at the highest salt concentration studied. That is, the application of 28 mg/L humic acid at 0.35 M salinity achieved an excellent success on the negative effect of salt stress, reaching to the same percentage (0.04) as the seeds germinated in distilled water. The important point here is that humic acid should be used at the appropriate concentrations, considering the negative effects on chromosome behaviors when it was used in non-stressed conditions. Humic acid application alone under non-stressed conditions may have functioned as a stimulator by triggering the synthesis of proteins necessary for normal cell division and by accelerating the mitotic cycle. The acceleration of the mitotic cycle may have led to a number of disruptions during the cell division and a significant increase of chromosomal abnormalities. As is known, external stimulator growth regulator applications are useless under normal conditions where there is no stress (Tabur and Demir 2010 a). Therefore, it is not surprising that the application of humic acid under stress conditions slows down mitotic activity in parallel with salt concentrations rise and eventually alleviating the negative effects of stress by regulating chromosome behaviors, and even removing (at 0.35 M salinity).

In addition, we can explain as follows the reason why various chromosomal aberrations observed during the microscopic examination of the root meristem cells of seeds belonging to all the applications: In general, accurate chromosome segregation in mitosis requires that sister kinetochores attach to microtubules emanating from opposite spindle poles. Because kinetochore attachment is a stochastic process, it is error prone and can result in chromosome malorientation (Rieder and Salmon 1998). Mitotic irregularities such as disorderly prophase, fault or distant polarization, alignment anaphase, vagrant chromosome and bridges may be mainly the result of the above mentioned reasons or spindle dysfunction. Generally, such abnormalities constitute a significant portion of chromosomal aberrations. The formations of micronuclei are likely the consequence of vagrant chromosomes and fragments. Also, some researchers reported that MNs, indicators of chromosomal genotoxicity and instability, are formed from one or more chromosomes (Bonciu et al. 2018). It is known that fragments are considered as structural changes in chromosomes and that chromosomes are affected by physical or chemical agents outside normal conditions (El-Ghamery et al. 2000). It has been reported that certain regions of chromosomes are broken by reacting with chemical substances and these regions are particularly heterochromatic regions (Rieger et al. 1973). Abnormal chromatin condensation expressed as chromatin granulation is concerned with the inhibition of enzymes and histone proteins. While laggard chromosomes could be the result of the failure of spindle apparatus to organize in normal way, sticky chromosomes may result from the improper folding of the chromatin fibres (Klášterská et al. 1976). According to some researchers, chromosomal stickiness is a marker of the toxic effect on chromatin (Fiskesjö and Levan 1993). The prophase and metaphase cells with uncoiled chromosomes may be due to disorderly chromosome contractions. The disrupted equatorial plate may result from unequal distribution of chromosomes and spindle dysfunction. Bonciu et al. (2018) asserted that nucleoplasmic bridges originate from dicentric chromosomes or occur as a result of a faulty longitudinal breakdown of sister chromatids during anaphase. It has also been reported that anaphase and telophase bridges may have been the result of inversions (Tabur and Demir 2010 b). It is thought that humic acid alone or salt concentrations used in our study may have been caused to all these abnormalities mentioned above by triggering the stimulation/ inhibition of enzymes and proteins necessary for the normal cell division, by disturbing the spindle mechanism and by accelerating mitotic cycle.

CONCLUSION

The mechanisms by which salinity inhibits growth are complex and controversial. Moreover, these mechanisms may vary substantially according to factors, such as plant species, the developmental stage of the plant, the strength of the stress and duration of the treatment. Unfortunately, a universal mechanism about this contradiction has not been established yet. Although the causes of salinity have been characterized, our understanding of the mechanisms by which salinity prevents plant growth is still rather poor. This work may provide new conceptual tools for designing hypotheses of salt tolerance in plants. As a result, we have attempted

to serve the filling of a gap in the literature by comparing their interactions between the mitotic activity and chromosome behaviors of humic acid under normal and salt stress conditions using barley seeds, an important model plant for molecular studies. In future studies, the investigation of the effects of humic acid on fundamental metabolic events such as nucleic acid metabolism, protein synthesis, and enzyme synthesis, which may be directly or indirectly effective on mitotic activity and chromosomal abnormalities will contribute to the clarification of mentioned mechanism.

ACKNOWLEDGEMENTS

We would like to thank Dr. Dilek Çavuşoğlu (SDU Atabey Vocational School, Food Business Administration) for their help in shaping. In addition, we are grateful to the Süleyman Demirel University Scientific Research Projects Management Unit for the financial support of this work.

FUNDING

This project is supported as financial by Süleyman Demirel University Scientific Research Projects Management Unit under Grant Project No. 3275-YL1-12.

REFERENCES

Anuradha S, Rao SSR. 2001. Effect of brassinosteroids on salinity stress induced inhibition of seed germination and seedling growth of rice (*Oryza sativa* L.). Plant Growth Regul. *33*(2): 151–153.

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

Calvo P, Nelson L, Kloepper JW. 2014. Agricultural uses of plant biostimulants. Plant Soil. 383: 3–41.

Canellas L, Olivares FL. 2014. Physiological responses to humic substances as plant growth promoter. Chem Biol Techn Agric. 1: 3.

Chen Y, Clapp CE, Magen H. 2004. Mechanisms of plant growth stimulation by humic substance: The role of organo-iron complex. Soil Sci Plant Nutr. 50(7): 1089–1095.

Çavuşoğlu K, Ergin H. 2015. Effects of humic acid pretreatment on some physiological and anatomical parameters of barley (*Hordeum vulgare* L.) exposed to salt stress. Bangladesh J Bot. 44(4): 591–598.

Çavuşoğlu D, Tabur S, Çavuşoğlu K. 2016. The effects of *Aloe vera* L. leaf extract on some physiological and cytogenetical parameters in *Allium cepa* L. seeds germinated under salt stress. Cytologia. 81(1): 103–110.

Dash M, Panda SK. 2001. Salt stress induced changes in growth and enzyme activities in germinating *Phaseolus muingo* seeds. Biol Plantarum. 44, 587–589.

du Jardin P. 2012. The science of plant biostimulants - A bibliographic analysis. Contract 30-CE0455515/00-96, Ad hoc study report on bio-stimulants products, http://hdl.handle.net/2268/169257, Accessed 20 April 2017.

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

El-Bassiouny HSM, Bakry BA, Attia AAE, Abd Allah MM. 2014. Physiological role of humic acid and nicotinamide on improving plant growth, yield, and mineral nutrient of wheat (*Triticum durum*) grown under newly reclaimed sandy soil. Agric Sci. 5: 687–700.

El-Ghamery AA, El-Nahas AI, Mansour MM. 2000. The action of atrazine herbicide as an indicator of cell divition on chromosomes and nucleic acids content in root meristems of *Allium cepa* and *Vicia faba*. Cytologia. 65: 277–287.

FAO 2015. Land and Plant Nutrition Management. http://www.fao.org/ag/agl/agll/spush/, Accessed 18 April 2017.

Feretti D, Ceretti E, Gustavino B, Zerbini I, Zani C, Monarca S, Rizzoni M. 2012. Ground and surface water for drinking: A laboratory study on genotoxicity using plant tests. J Public Health Res. 1: 31–37.

Ferrara G, Loffredo E, Simeone R, Senesi N. 2000. Evaluation of antimutagenic and desmutagenic effects of humic and fulvic acids on root tips of *Vicia faba*. Environ Toxicol. 15: 513–517.

Fiskesjö G, Levan A. 1993. Evaluation of the first ten MEIC chemicals in the *Allium* test. ATLA. 21: 139–149.

Gichner T, Badaev F, Pospisil F, Veleminsky J. 1990. Effects of humic acids, para-aminobenzoic acid and ascorbic acid on the n-nitrosation of the carbamate insecticide propoxur and on the mutagenicity of nitrosopropoxur. Mut Res. 229: 37–41.

Gorova A, Skvortsova T, Klimkina I, Pavlichenko A. 2005. Cytogenetıc effects of humic substances and their use for remediation of polluted environments. In: Perminova IV, Hatfield K, Hertkorn N. (Eds.), Use of Humic Substances to Remediate Polluted

Environments: From Theory to Practice, Netherlands: Springer. p. 311–328.

Jindo K, Martim SA, Navarro EC, Pérez-Alfocea F, Hernandez T, Garcia C, Aguiar NA, Canellas LP. 2012. Root growth promotion by humic acids from composed and non-composed urban organic wastes. Plant Soil. 353(1–2): 209–220.

Khaled H, Fawy HA. 2011. Effect of different levels of humic acids on the nutrient content, plant growth, and soil proerties under conditions of salinity. Soil Water Res. 6(1): 21–29.

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

Lutsenko EK, Marushko EA, Kononenko NV, Leonova TG. 2005. Effects of fusicoccin on the early stages of sorghum growth at high NaCl concentrations. Russ J Plant Physiol. 52: 332–337.

Miller G, Suzuki N, Çiftci-Yılmaz S, Mittler R. 2010. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant Cell Environ. 33: 453–467.

Munns R, Tester M. 2008. Mechanisms of salinity tolerance. Annu Rev Plant Biol. 59: 651–681.

Rieder CL, Salmon ED. 1998. The vertebrate cell kinetochore and its roles during mitosis. Trends Cell Biol. 8: 310-318.

Rieger R, Nicoloff H, Michaelis A. 1973. Introchromosomal clostering of chromatic aberrations induced by N-Methyl-N-Nitroso urethan in *Vicia faba* and barley. Biol Zent. 92: 681–689.

Sharma PC, Gupta PK. 1982. Karyotypes in some pulse crops. Nucleus. 25: 181-185.

Sivananthi T, Paul AJ. 2014. Effect of humic acid of vermicompost on *Zea mays* root growth. SIRJ-APBBP. 1(2): 7–16.

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

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

Tajbakhsh M, Zhou MX, Chen ZH, Mendham NJ. 2006. Physiological and cytological response of salt-tolerant and non-tolerant barley to salinity during germination and early growth. Aust J Exp Agr. 46(4): 555–562.

Wang W, Vinocur B, Altman A. 2003. Plant responses to drought, salinity and extreme temperatures: Towards genetic engineering for stress tolerance. Planta. 218: 1–14.

Zhu M, Shabala S, Shabala L, Fan Y, Zhou MX. 2016. Evaluating predictive values of various physiological indices for salinity stress tolerance in wheat. J Agron Crop Sci. 202: 115–124.

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: A. Naik, A.K. Patel, S.K. Mishra, A. Nag, J. Panigrahi (2019) Characterization of intraspecific hybrid in *Clitoria ternatea* (L.) using morpho-physiological, cytogenetic, metabolic and molecular markers. *Caryologia* 72(3): 11-22. doi: 10.13128/caryologia-754

Published: December 13, 2019

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

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

Characterization of intraspecific hybrid in *Clitoria ternatea* (L.) using morpho-physiological, cytogenetic, metabolic and molecular markers

Aparupa Naik[1], Amiya K. Patel[1], Sujit K. Mishra[1], Atul Nag[2], Jogeswar Panigrahi[1,2,*]

[1] *Plant Biotechnology Laboratory, Department of Biotechnology and Bionformatics, Sambalpur University, Jyoti Vihar-768019, Sambalpur, Odisha, India*
[2] *Department of Biotechnology, Central University of Rajasthan, NH-8, Bandarsindri, Kishangarh, Rajasthan*
*Corresponding author: drjpanigrahi@gmail.com

Abstract. *Clitoria ternatea* (L.) is a medicinal plant possessed with bioactive molecules such as taraxerol, delphinidin, kaempoferol and quercetin etc. For the development genotype with higher content of these bioactive molecules, marker-assisted breeding is one of the best strategies and it initiates with the development of F_1 hybrids. Thus, an intraspecific F_1 hybrid was raised involving two contrasting genotypes of *C. ternatea acc. CtB3-SL1* (Blue flowered) and *C. ternatea acc. CtW2-BL1* (white flowered). The hybridity of the F_1 plant was confirmed by assessing the phenotypic traits, such as colour of the petal, pod shape and seed coat colour, 100 seed weight, and the content of taraxerol and delphinidin. The pollen mother cells in the F_1 hybrid showed eight bivalents with preponderance of ring bivalents and 8I:8I segregation at metaphase-I and Anaphase-I, respectively. SDS-PAGE seed albumin and globulin detected three pollen parent-specific polypeptides (Mw 31.62, 22.38 and 18.81KDa), and were inherited to F_1 hybrids, which evidenced the hybridity of putative F_1 plants. Further, DNA marker analysis also showed the inheritance of 11 RAPD, six SCoT and one ISSR markers to putative F_1 plant, which affirmed the hybrid nature of the F_1 plant. This study also evidenced that combined use of morphophysiological, cytogenetic, protein and DNA marker analyses could be effective for precise characterization of intra-specific hybrids in *C. ternatea*. These F_1 hybrid and its derived future progenies could also be used for mapping of QTLs or genes contributing higher accumulation of taraxerol and delphinidin in different plant parts.

Keywords. Hybridity, Meiosis, Seed protein profile, DNA marker, Taraxerol, Delphinidin.

INTRODUCTION

Clitoria ternatea (L.) belongs to the family-Leguminosae (Fabaceae) with somatic chromosome count 2n=2x=16 and was distributed worldwide

(Joson and Ramirez 1991; NPGS2008). The *C. ternatea* is also known as Shankhpushpi, Butterfly pea, Aparajita, Gokarni, Girikarnika (Bishoyi et al. 2014). The plant is elongated, slender, climbing herbaceous vine with five leaflets, white to purple flowers, and has deep roots. Butterfly pea is predominantly a self-pollinated species, but sometimes considered as often cross-pollinated due to the appearance of segregating genotypes in some populations (Cook et al. 2005). This plant has been considered as important medicinal plant, and the phyto-chemical studies explored several bioactive metabolites such as alkaloids, triterpenoids, flavonoids, glycosides, anthocyanins and lactones etc. in this species (Mukherjee et al. 2008; Sethiya et al. 2009). Thus the plant parts based extracts have been prepared and used as a memory enhancer, nootropic, anti-stress, anxiolytic, anticonvulsant, tranquilizing and sedative agent (Parrotta 2001; Prajapati et al. 2003; Khare 2004; Kapoor 2005; Margret et al. 2015). Among the bioactive metabolites, content of kaempferol, delphinidin and taraxerol having anti-cancer and anti-tumour properties have also been documented in this species (Braig et al. 2005; Chen and Kong 2005; Niering et al. 2005; Singletary et al. 2007; Swain et al. 2012). The white-flowered genotypes of *C. ternatea* were found to be medicinally rich in taraxerol and kaempferol, whereas the blue flowered genotypes were affluent in delphinidin, quercetin and isoquercetin etc. For the development of improved genotype with higher content of these bioactive metabolites marker assisted breeding (MAB) is one of the finest strategy, where F_1 hybrid serves as the starting material for all future breeding efforts for the genetic improvement. Thus intra-specific F_1 hybrid involving blue flowered and white flowered genotype can be used as the starting material for widening genetic base of this medicinal species in term of bioactive metabolites composition and production of advanced breeding lines containing desired metabolites of pharmaceutical importance. However, interaction between both the genome in either of the parental cytoplasmic background often led to genetic variation due to genetic recombination, differential gene action, penetrance and expressivity. F_1 hybrid being the starting material for all breeding efforts, its precise identification at early stage is mandatory. Thus morphological, cytological, biochemical and molecular markers have been effectively used to ascertain hybridity of F_1 plants in many species. But the reproducibility of morphological, cytological and biochemical markers in consonance to environmental variation and developmental regulations limits their applicability. Therefore seed protein and DNA sequence based marker analysis could also be utilized to screen and identify F_1 hybrids at an early stage because of their stability, uniformity, reliability and reproducibility across the environment and are also free from penetrance and expressivity.

The seed protein markers have been effectively employed for cultivar characterization, genetic diversity assessment, and verification of hybridity in many species (Mohanty et al. 2001; Panigrahi et al. 2007; Jisha et al. 2011; Mishra et al. 2012). Similarly DNA markers, such RAPD, ISSR and SSR have been used for characterization of hybrids in different species (Lima-Brito et al. 2006; Muthusamy et al. 2008; Goldmann et al. 2008; Hemalatha et al. 2010; Bianco et al. 2011; Mishra et al. 2012; Mishra et al. 2017). In case of *C. ternatea*, few of these DNA markers have only been used for genetic diversity studies (Chandra 2011; Swati et al. 2011; Ganie et al. 2012; Ali et al. 2013: Bishoyi et al. 2014). However, no report has been made so far on the development of intra-specific hybrid in *C. ternatea* and its characterization.

In the present study, an intra-specific F_1 hybrid of *C. ternatea*, involving blue flowered [*C. ternatea acc. CtB3-SL1*] and white flowered [*C. ternatea acc. CtW2-BL1*] genotype, was raised and its hybridity was affirmed by simultaneous use of morpho-physiological and cytogenetic analyses, estimation of bioactive metabolite, profiling of seed protein and DNA based markers.

MATERIALS AND METHODS

Plant materials

Genotypes of *C. ternatea* (including of five white genotypes, six blue genotypes, and four bipetaloid blue genotypes) were collected from Sambalpur and Bargarh districts of Odisha and maintained at the experimental garden, School of Life Sciences, Sambalpur University, Odisha, India (Table 1). Among these genotypes, two genotypes of *C. ternatea acc. CtB3-SL1* and *C. ternatea acc. CtW2-BL1* were identified on the basis of their bioactive metabolites (Delphinidin and Taraxerol) content, and used as seed parent and pollen parent, respectively for the development of F_1 hybrid.

Morpho-physiological traits characterization

Different morpho-physiological traits like colour of standard petal, flower length, flower breadth, floral bud size, anther size, style length, stigma length, seed coat colour and 100 seeds weight were studied for three F_1 plants along with their parents. The morpho-physiological traits unique to pollen parent were used as visual DUS marker for the characterization of F_1 hybrid.

Table 1. Fourteen accessions of *C. ternatea* with their flower colour, petal configuration and the geographical coordinates of collection sites located in Odisha, India.

Sl. no.	Accession (acc._)	Collection Site	Latitude	Longitude	Mean Sea Level(m)	Flower Colour & Petal structure
1	CtW1-BG1	Bargarh	21°22'50"N	83°44'48"E	186	White unipetaloid
2	**CtW2-BL1**	**Sriram vihar (Burla)**	**21°28'46"N**	**83°53'5"E**	**172**	**White unipetaloid**
3	CtW3-BL2	Burla Town	21°28'46"N	83°53'5"E	172	White unipetaloid
4	CtW4-BGK1	Kandahata (Bargarh)	21°15'57"N	83°39'54"E	186	White unipetaloid
5	CtW5-BG2	Bargarh	21°22'50"N	21°22'50"E	186	White unipetaloid
6	CtW6-BG3	Bargarh	21°22'50"N	21°22'50"E	186	White unipetaloid
7	CtB1-KL1	kuchinda	21°37'34"N	83°19'0"E	254	Blue unipetaloid
8	CtB2-BGK2	Kandahata (Bargarh)	21°15'57"N	83°39'48"E	186	Blue unipetaloid
9	**CtB3-SL1**	**Sambalpur**	**21°46'81"N**	**83°97'54"E**	**151**	**Blue unipetaloid**
10	CtB4-PL1	Padampur (Bargarh)	21°0'0"N	83°3'46"E	205	Blue unipetaloid
11	CtB5-PL2	Padampur (Bargarh)	21°0'0"N	83°3'46"E	205	Blue unipetaloid
12	CtBB1-PL3	Padampur (Bargarh)	21°0'0"N	83°3'46"E	205	Blue bi-petaloid
13	CtBB-PL4	Padampur (Bargarh)	21°0'0"N	83°3'46"E	205	Blue bi-petaloid
14	CtBB-PL5	Padampur (Bargarh)	21°0'0"N	83°3'46"E	205	Blue bi-petaloid

Cyto-genetic characterization

To study the chromosome homology, mitotic and meiotic analysis of F_1hybrids and their parents were carried out following Behera et al. (2010). Well-developed roots (1-3 cm) obtained from the seedlings at 8.00-8.30 a.m. and incubated with pre-chilled p-dichlorobenzene (PDB) solution for two hours at 20°C, followed by fixation in 1:3 aceto-alcohol and kept overnight at room temperature. Subsequently, the root tips were transferred to 70% ethanol and stored at 4^0C. Hydrolysis of the root tips was carried out in preheated 1N HCl at 60^0C for 10 min followed by staining with the help of 1.5% aceto-orcein for one hour and squashed with 45% propionic acid. For meiotic analysis, the flower buds of appropriate size were fixed in 1:3 aceto-alcohol and kept overnight at 25 ± 2°C and then it was transferred to 70% ethanol and stored at 4°C. The anthers of suitable size were squashed in a drop of 1.5% acetocarmine, and the meiotic behaviour of chromosomes at diakinesis, metaphase-I and anaphase-I were observed. Suitable stages of mitosis and meiosis were observed under a compound microscope (Unilab, India) and were documented using Nikon Coolpix-4500 camera.

Bioactive metabolites characterization

Estimation of taraxerol in root tissues: Roots of *C. ternatea* genotypes and their F_1 hybrid were collected after 30 days of initiation of flowering, air dried under shade and ground to fine powder. Powder of each sample (appx. 20gm) was subjected to extraction with 70% alcohol for 5h at 60°C, filtered (using Whatmann No.1 filter paper) and dried under vacuum in a rotary evaporator (RV-10, IKA, Germany). The hydroalcoholic extract (appx. 8.4 g) was suspended in water and sequentially extracted using hexane, chloroform, ethyl acetate and n-butanol as described by Kumar et al. (2008). The hexane and chloroform fractions were subjected to chromatography using Chlorofom: methanol (1:1, v/v) as eluent and the eluted fractions were further chromtographed using hexane: ethyl acetate (80:20, v/v) as eluent and yielded the delphinidin as described earlier (Kumar et al. 2008). This compound was dissolved in ethanol (1mg.ml^{-1}) and 10 µl aliquot of each sample was used for HPTLC assay along with standard taraxerol solution (10-100µg.ml^{-1}) as described by Kumar et al. (2008). Thin layer chromatography was carried out using aluminum backed HPTLC plates (100cm^2; 0.2 mm thickness) of silica gel 60 F_{254} (Merck, Germany) in a HPTLC system (CAMAG, Switzerland) consisting of Linomat-IV sampler, twin plate development chamber and CAMAG TLC scanner 3 with WINCATS software. The derivatized plates were scanned under visible light and the content of taraxerol was estimated densitometrically by measuring absorption at 420 nm by TLC scanner-3 integrated with WINCATS v 1.4.2 software (slit dimension- 6 mm x 0.45 mm; scanning speed- 20 mm.s^{-1}).

Estimation of delphinidin in flowers: The flowers were collected, air dried and extracts of petals were prepared following Fukui et al. (2003). The petal

extracts were dissolved in 0.2 ml of 6 N HCl and kept at 100 °C for 20 min. The hydrolyzed anthocyanidins were extracted with 0.2 ml of 1-pentanol. HPLC was performed using an ODS-A312 column as described by Katsumoto et al. (2007) using acetic acid : methanol : water (15 : 20 : 65) as solvent with flow rate 1ml per min, and the delphinidin content was estimated by measuring absorbance from 400-600 nm on photodiode array detector (SPD-M10A; Shimadzu Co., Ltd). Under these HPLC conditions, the λ_{max} of delphinidin and retention time were 540 nm and 4 min, respectively which were validated with those of delphinidin chloride (Sigma-Aldrich).

Proteomic characterization using seed protein profiling

The albumin and globulin fraction of the seeds were extracted and denatured as described by Panigrahi et al. (2007). Protein samples (appx. 25.0 µg) were separated under discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli et al. 1970) using 10 % resolving gel (0.375 M Tris–HCl, pH 8.8) and 4% stacking gel (0.125 M Tris–HCl, pH 6.8). Tris-glycine (0.1% SDS, 25 mM Tris-glycine, pH 8.3) was used as running buffer, and electrophoresis was carried out at 1.5 mA per well constant current until tracking dye reaches the separating gel, and then current supply was increased to 2 mA per well till tracking dye reach bottom of the gel. The molecular weight marker-PMWM (Genei Pvt. Ltd.) was used as a standard, and the size of polypeptides was estimated by standard curve method.

Genomic characterization using DNA markers

The genomic DNA from F_1 hybrid and its parents were isolated using the modified CTAB method (Sivaramakrishnan et al. 1997) and purified (Mishra et al. 2012). DNA was dissolved in 2.0 ml TE (Tris-EDTA) buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and stored at -20°C. Purity and concentration of the DNA sample were measured using a UV-Vis spectrophotometer (UV 1601, Shimadzu, Kyoto, Japan) by taking TE buffer as the blank. The quantification of DNA was validated by analyzing the purified DNA on 0.8 % (w/v) agarose gel by taking diluted, uncut phage lambda DNA as standard. The DNA samples were equilibrated to 10 ngµl^{-1} in TE buffer. For RAPD, SCoT and ISSR marker analysis, the PCR amplification of 25ng of genomic DNA was carried out using 30 random decamer oligonucleotide primers (OPA-01-20 and OPB-01-10; Operon Technologies, Alameda, CA, USA), 36 SCoT primers (SCoT -1 to SCoT -36; Collard and Mackill 2009) and seven ISSR primers from the set 100/9 (UBC-861, UBC-865, UBC-868, UBC-873, UBC-872, UBC-808, UBC-807; University of British Columbia, Vancouver, Canada), respectively. The PCR amplification reaction (25µl) contained 25 ng template DNA, 2.5µl of 10X assay buffer [100 mM Tris-Cl, pH 8.3; 0.5 M KCl; 0.1 % (w/v) gelatin], 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.25µM primer, 1.0 units *Taq* DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India). For RAPD, ISSR and SCoT analysis, the amplification were carried out using a thermal cycler (GENEAMP-9700; Applied Biosystems, Foster City, USA), and conditions are as below:

Amplification Conditions	RAPD	ISSR	SCoT
Initial Denaturation	94 °C for 5 min		
PCR cycles	45 cycles	40 cycles	35 cycles
Cyclic Denaturation	94 °C for 60 s	94 °C for 30 s	94 °C for 30 s
Annealing of primers	37 °C for 60 s	40-60 °C for 60 s	50 °C for 60 s
Elongation	72 °C for 2 min	72 °C for 2 min	72 °C for 2 min
Final Elongation	72 °C for 5 min	72 °C for 5 min	72 °C for 5 min
Storage of sample	4 °C for ∞ min		

The amplified products were mixed with gel loading buffer [20 % (w/v) sucrose; 0.1 M EDTA, 1.0 % (w/v) SDS; 0.25 % (w/v) bromo-phenol blue; 0.25 % (w/ v) xylene cyanol] and separated in 1.4 % (w/v) agarose gel containing 0.5µgml^{-1} ethidium bromide in TAE buffer (40 mM Tris acetate, pH 8.0; 2 mM EDTA) at 50V constantly. The separated DNA fragments were documented using gel documentation system (Gel Doc XR system, Biorad, USA), size of amplified fragments was estimated using TL-120 software (Non-linear Dynamics, Total Lab Ltd., Newcastle Upon Tyne, UK) and 250 bp step-up ladder (Bangalore Genei Pvt. Ltd.) as standard.

RESULTS

In this study, the putative intra-specific F_1 hybrids were raised by convetional hybridization and were characterized by using morpho-physiological and cytogenetic analysis, estimation of bioactive metabolites, seed protein (albumin and globulin) profiling and DNA marker analysis.

Morpho-physiological and metabolite characterization of the intraspecific F_1 hybrid

Morpho-physiological traits including flower colour, pod beak, and seed coat colour distinguish the blue flowered parental genotype *C. ternatea* (*acc. CtB3-SL1*) from white flowered one (*C. ternatea acc. CtW2-BL1*). The size of flower and 100 seed weight were also distinguishes both the parents. In many such morpho-physiological traits, the F_1 hybrid was intermediate between the parents with predominance of the characters of *C. ternatea*, *acc. CtW2-BL1*, such as seed coat colour, petal colour being the pollen parent, and their appearance in the intermediate form was also very vivid for the identification of the F_1 hybrid (Table 2; Fig. 1a, b). In most of the quantitative traits, such as leaf size, flower size, and 100 seed weight, the F_1 plant resided well around the mid-parental value (Table 2).

The raised F_1 hybrids were assessed along with their parents for two important bioactive metabolites (Taraxerol and Delphinidin). Taraxerol was obtained mainly from root tissues whereas delphinidin was obtained from the petals of the flowers. The F_1 hybrid contains 0.856±0.031 mg.g^{-1} taraxerol in its root tissue and 0.372±0.019 mg.g^{-1} delphinidin in its flowers. On comparison with its parents taraxerol content in root tissue of F_1 hybrid was almost at par with the donor parent (*C. ternatea acc. CtW2-BL1*) whereas delphinidin content was intermediate between both the parents (Table 2; Fig. 1c).

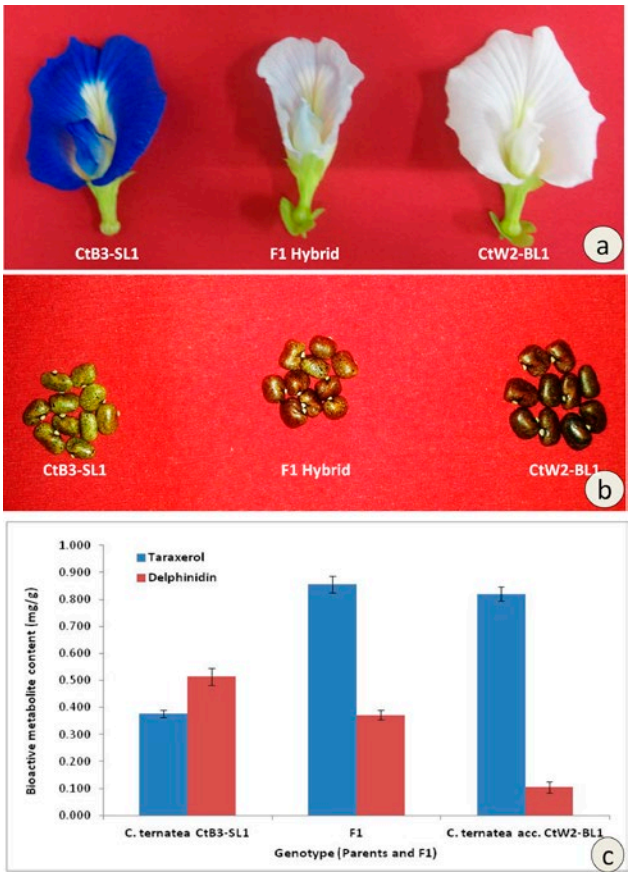


Fig. 1. Characterization of intra-specific F_1 hybrid along with its parents (*C. ternatea acc. CtB3-SL1* and *C. ternatea acc. CtW2-BL1*). Floral attributes including petal colour (a), Mature seeds showing colour of the seed coat and aril (b); Graphical representation of bioactive metabolites (taraxerol and delphinidin) content.

Cyto-genetical characterization of the intraspecific F_1 hybrid

Appropriate stages like metaphase, anaphase, diakinesis, metaphase-I, anaphase-I were observed in the

Table 2. Morpho-physiological and metabolite characterization of *intra-specific F_1* hybrid of *C. ternatea.*

Morphological traits	*C. ternatea acc. CtW2-BL1*	*C. ternatea acc. CtB3-SL1*	F_1 hybrid
Shape of the leaflets	Lanceolate	Lanceolate	Ovate-lanceolate
Base of the leaflets	Cuneate	Oblique	Oblique
Flower length (cm)	5.23±0.15	5.03±0.15	5.08±0.1
Flower breadth (cm)	3.2±0.1	3.04±0.06	3.07±0.06
Average days to flowering	56 days	64 days	64 days
Colour of the Petal	White	Blue	Intermediate
Nature of the ovary & style	Pubescent	Glabrous	Glabrescent
Seed colour	Black	Brown	Blakish brown
Number of seeds per pod	4-5	5-6	4-5
100 Seed weight (g)	5.82 ± 0.19	5.16 ± 0.2	5.26 ± 0.18
Taraxerol Content (mg.g^{-1})	0.821 ± 0.026	0.377 ± 0.014	0.856 ± 0.031
Delphinidin Content (mg.g^{-1})	0.104 ± 0.02	0.514 ± 0.019	0.372 ± 0.019

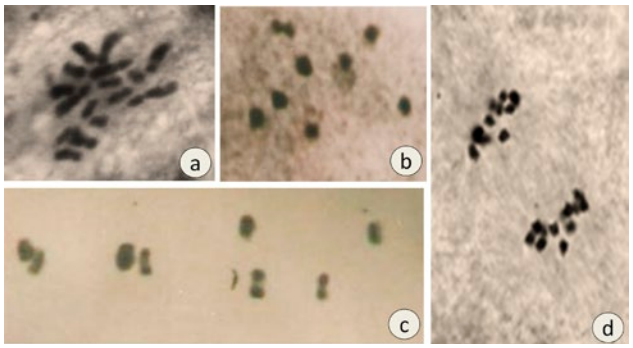


Fig. 2. Cytogenetic characterization of F_1 hybrid showing 2n=2x=16 chromosome configuration at mitotic metaphase (a), association of eight bivalents at diakinesis (b) and metaphase-I (c), and segregation of chromosome at anaphase-I (d).

PMCs of both the parents and F_1 hybrid. Chromosome analysis of both the parents revealed 16 chromosomes at metaphase in each of them, and the separation at anaphase occurs in a normal fashion. The mitotic metaphase of F_1 hybrid showed 16 distinct chromosomes similar to its parents (Fig. 2a). As expected, the PMCs of the F_1 hybrid showed formation of eight bivalents (II) at diakinesis and metaphase-I (Fig. 2b, c) and 8II: 8II separation at anaphase-I (Fig. 2d). The pollen fertility in the F_1 hybrid was almost 86% and was equivalent to its parents.

Proteomic and genomic characterization of the intraspecific F_1 hybrid

SDS-PAGE of seed albumins of two parental genotypes, including *C. ternatea acc. CtB3-SL1* and *acc. CtW2-BL1,* and their F_1 hybrids led to the detection of 33 polypeptide bands with molecular weight 12.59 to 84.14 KDa**.** Out of which 30 polypeptides were monomorphic, and rest three were varied for their expression (Table 3; Fig. 3). The putative F_1 hybrid possessed with three polymorphic polypeptides (Mw 31.62, 22.38 and 18.81 KDa) specific to pollen parent *C. ternatea acc. CtW2-BL1* along with the monomorphic polypeptides (Table 3; Fig. 3). Since *C. ternatea acc. CtW2-BL1* was used as pollen parent, the appearance of these unique albumin polypeptides in the F_1 hybrid can potentially be used as markers for identification of hybrids involving at least *C. ternatea acc. CtW2-BL1* as pollen parent.

In the present study, all three kinds of DNA markers showed polymorphism *at par.* Thirty RAPD primers have amplified 127 RAPD fragments ranging from 130 to 2389 bp, and among them, 22 amplified fragments (17.32%) showed parental polymorphism (Table 4). Contrasting to this amplification with nine ISSR and

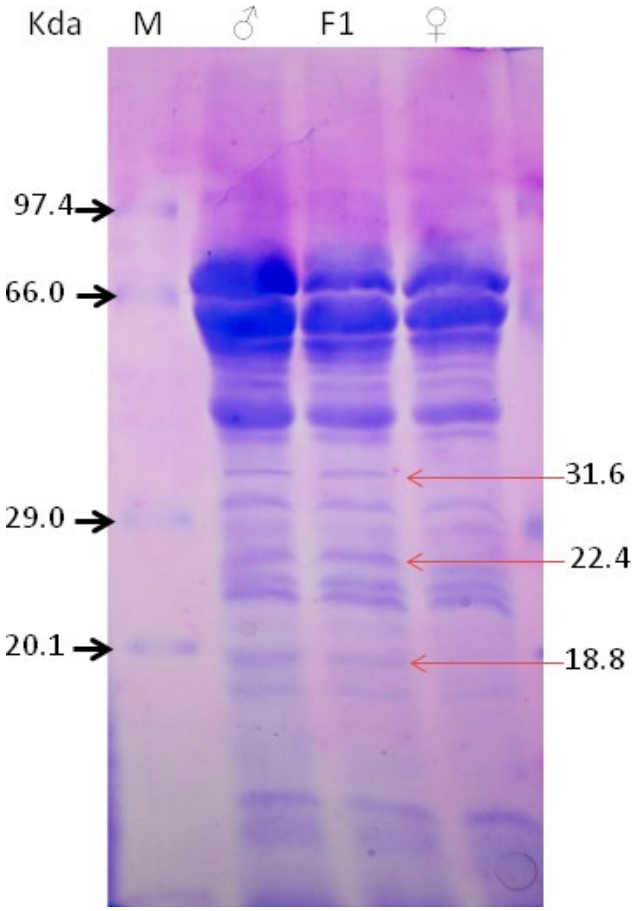


Fig. 3. Inheritance of seed albumin markers to intra-specific F_1 hybrid (*C. ternatea acc. CtB3-SL1* X *C. ternatea acc. CtW2-BL1*) intra-specific F_1 hybrid; Lane 'M' represents Mol. Weight Marker (PMW-M, GENEI, India), and arrow indicates on right hand side indicate the polypeptides inherited to the F_1 hybrid (MW in kDa).

36 SCoT primers generated 39 and 224 fragments ranging from 437 to 3154 bp and 116 to 3916 bp, respectively (Table 4, 5). Both ISSR and SCoT analysis showed lower polymorphism (2.56% and 5.80%) in comparison to RAPD markers. Among the parental polymorphic markers 22 RAPD, one ISSR and 13 SCoT markers were inherited to the putative F_1 hybrid, and among them 11 RAPD and six SCoT markers, unique to pollen parent (*CtW2-BL1*), were very vivid in its appearance for the identification of F_1 hybrid (Fig. 4). The total number of fragments amplified, percentage of polymorphism, inheritance of polymorphic fragments to the F_1 hybrid were shown in Table 4 and 5**.**

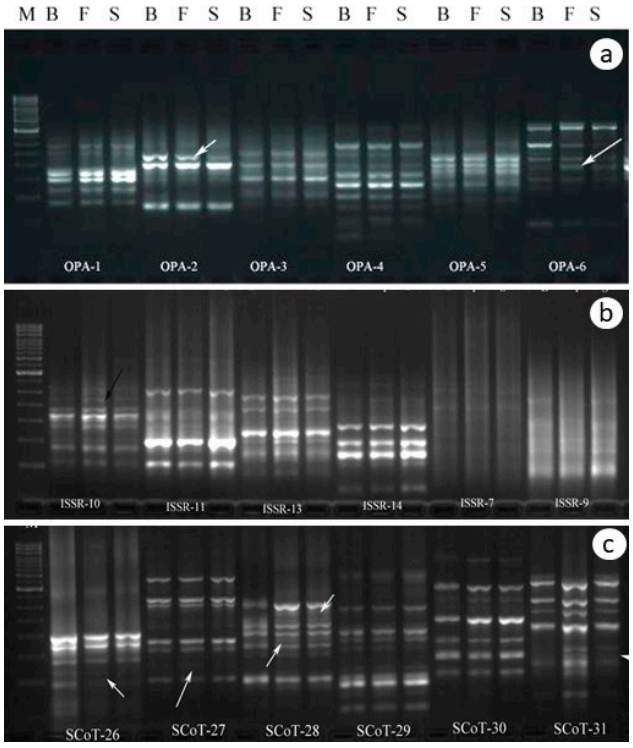


Fig. 4. Inheritance of pollen parent specific fragments (→) to *C. ternatea acc. CtB3-SL1* X *C. ternatea acc. CtW2-BL1* intra-specific F_1 hybrid, generated by RAPD primers (a), ISSR primers (b) and SCoT primers (c). (M: 250 bp step-up ladder, B: *C. ternatea acc. CtW2-BL1*, F: F_1 hybrid and S: *C. ternatea acc. CtB3-SL1*).

DISCUSSION

The F_1 hybrids have been considered as starting material for all breeding endeavours including the development of mapping population, mapping, and tagging of traits, marker-aided selection and generation of advanced breeding lines before the release of cultivars. Thus, identification and characterization of F_1 hybrids at an early stage during hybridization programme is quite essential (Lima-Brito et al. 2006; Mishra et al. 2012). In this study, the putative intra-specific F_1 hybrid was characterized by using morpho-physiological and cytogenetic analyses, estimation of bioactive metabolites (taraxerol and delphinidin), seed protein (albumin and globulin) profiling, and DNA marker analysis.

The F_1 hybrid was intermediate between the parents (*C. ternatea acc. CtB3-SL1* and *acc. CtW2-BL1*),in term of morpho-physiological traits distinguish the parents, such as flower colour, pod beak and seed coat color, size of flower and 100 seed weight. Although no pervious report is available in *C. ternatea*, in several species the character(s) distinguish the parents were appeared in its intermediate form in F_1 hybrid (Mishra et al. 2012; Mishra et al. 2017) and in some cases the pollen parent specific traits were also predominated as observed in the present study. Either the appearance of pollen parent specific traits in the F_1 or appearance of traits in intermediate form could be used vividly for the identification of hybridity. The consistency of metabolites in the F_1 hybrid of any medicinal plant in consonance to their parents is very vital in the perspective of their use as source material either to harvest therapeutics compounds or to generate breeding lines. *C. ternatea* plant parts are source of many important bioactive metabolites, and also have wide range of biological and pharmacological activities (Mukherjee et al. 2008; Sethiya et al. 2009). In view of this, the raised F_1 plants were assessed along with their parents for two important bioactive metabolites, Taraxerol and Delphinidin, mostly used for the treatment of various kind cancer and tumours (Braig et al. 2005; Chen and Kong 2005; Niering et al. 2005; Singletary et al. 2007; Swain et al. 2012). Taraxerol was obtained mainly from root tissues whereas delphinidin was obtained from the petals of the flowers. The F_1 hybrid contains 0.856±0.031 mg/g Taraxerol in its root tissue and 0.372±0.019 mg/g delphinidin in its flowers. On comparison with its parents taraxerol content in root tissue of F_1 hybrid was almost at par with the pollen parent (0.821±0.026) whereas delphinidin content was intermediate between both the parents (*C. ternatea acc.*

Table 3. Details of seed albumin and seed globulin markers inherited to the intraspecific F_1 hybrid.

Marker	Total no of bands	Range of Molecular Weight (KDa)	No. of polymorphic polypeptides	No. of polymorphic bands inherited to F_1 from		Parents specific bands in F_1 from (kDa)	
				CtW2-BL1	*CtB3-SL1*	*CtW2-BL1*	*CtB3-SL1*
Seed albumin	20	14.13-66.83	03 (15.0%)	03	--	**31.6, 22.4 & 18.8**	--
Seed globulin	13	12.59-84.14	--	--	--	--	--
Total	**33**	**13.34-112.2**	**03 (9.09%)**	**03**	**--**	**31.6, 22.4 & 18.8**	

Table 4. Details of RAPD and ISSR markers used for characterization of intraspecific F_1 hybrid showing the inheritance of parent specific markers to the intraspecific F_1 hybrid.

Primer	Sequence (5 ' → 3')	No. of fragments amplified	Range of amplified fragments (bp)	Polymorphic bands	Percentage of polymor-phism (%)	No. of Non-parental fragments* in F_1	Parent specific polymorphic bands (bp) in F_1 from	
							CtW2-BL1	*CtB3-SL1*
RAPD Marker								
OPA-01	CAGGCCCTTC	7	176-1500	---	--	$OPA1_{1500}$	---	---
OPA-02	TGCCGAGCTG	3	432-1233	$OPA-02_{1233}$	33.33	---	---	$OPA-02_{1233}$
OPA-03	AGTCAGCCAC	4	273-968	---	0	---	---	---
OPA-04	AATCGGGCTG	9	170-1500	$OPA-04_{170}$ $OPA-04_{314}$	22.22	---	$OPA-04_{170}$	$OPA-04_{314}$
OPA-05	AGGGGTCTTG	6	506-1233	$OPA-05_{506}$	16.66	---	---	$OPA-05_{506}$
OPA-06	GGTCCCTGAC	8	276-2045	$OPA-06_{1516}$ $OPA-06_{612}$	25	---	---	$OPA-06_{1516}$ $OPA-06_{612}$
OPA-07	GAAACGGGTG	5	530-1937	---	---	---	---	---
OPA-08	GTGACGTAGG	4	130-1019	$OPA-07_{895}$	25	---	$OPA-07_{895}$	---
OPA-09	GGGTAACGCC	5	461-1401	---	---	---	---	---
OPA-10	GTGATCGCAG	2	277-911	---	---	---	---	---
OPA-11	CAATCGCCGT	6	139-2389	$OPA-11_{1079}$	16.66	---	$OPA-11_{1079}$	---
OPA-12	TCGGCGATAG	5	330-2333	$OPA-12_{2333}$	20	---	$OPA-12_{2333}$	---
OPA-13	CAGCACCCAC	3	758-1144	---	---	---	---	---
OPA-14	TCTGTGCTGG	2	599-717	---	---	---	---	---
OPA-15	TTCCGAACCC	9	284-2250	$OPA-15_{2250}$ $OPA-15_{622}$	22.22	---	$OPA-15_{2250}$ $OPA-15_{622}$	---
OPA-16	AGCCAGCGAA	9	299-2187	$OPA-16_{2167}$ $OPA-16_{622}$	22.22	---	$OPA-16_{2167}$	$OPA-16_{622}$
OPA-17	GACCGCTTGT	1	437	---	0	---	---	---
OPA-18	AGGTGACCGT	2	569-974	$OPA-18_{974}$	50	---	$OPA-18_{974}$	---
OPA-19	CAAACGTCGG	1	1193	$OPA-19_{1193}$	100	---	---	$OPA-19_{1193}$
OPA-20	GTTGCGATCC	1	1000	---	0	---	---	---
OPB-01	GTTTCGCTCC	3	777-1417	$OPB-01_{1417}$ $OPB-01_{976}$	66.66	---	$OPB-01_{1417}$ $OPB-01_{976}$	---
OPB-02	TGATCCCTGG	2	759-898	---	---	---	---	---
OPB-03	CATCCCCCTG	2	637-1689	---	---	---	---	---
OPB-04	GGACTGGAGT	2	942-2187	---	---	---	---	---
OPB-05	TGCGCCCTTC	6	539-1653	---	---	$OPB-05_{539}$	---	---
OPB-06	TGCTCTGCCC	6	750-1575	---	0	--	---	---
OPB-07	GGTGACACGG	2	741-1377	$OPB-07_{741}$	50	---	$OPB-07_{741}$	---
OPB-08	GTCCACACGG	8	520-1520	$OPB-08_{1386}$ $OPB-08_{1000}$ $OPB-08_{870}$ $OPB-08_{520}$	50	$OPB-09_{1530}$ $OPB-09_{1164}$	---	$OPB-08_{1386}$ $OPB-08_{1000}$ $OPB-08_{870}$ $OPB-08_{520}$
OPB-09	TGGGGGACTC	0	---	---	0	---	---	---
OPB-10	CTGCTGGGAC	4	956-2156	---	0	---	---	---
Total		**127**	**130-2389**	**22**	**17.32**	**4**	**11**	**11**
ISSR Marker								
UBC-861	$(ACC)_6$	4	---	---	---	---	---	---
UBC-865	$(CCG)_6$	6	445-1000	---	---	---	---	---
UBC-868	$(GAA)_6$	10	539-1889	$UBC-868_{1486}$	10.0	---	---	$UBC-868_{1486}$
UBC-873	$(GACA)_4$	6	505-1541	---	---	---	---	---
UBC-872	$(GATA)_4$	2	1250-3038	---	---	---	---	---
UBC-808	$(AG)_8C$	12	429-2036	---	--	$UBC808_{1058}$ $UBC808_{981}$	---	---
UBC-807	$(AG)_8T$	3	592-924	---	---	---	---	---
		39	**437-3154**	**2**	**2.56**	**2**	**0**	**1**

Table. 5. Details of SCoT markers used for characterization of intraspecific F_1 hybrid showing the inheritance of parent specific markers to the intraspecific F_1 hybrid.

Primer	Sequence (5 ' → 3')	No. of fragments amplified	Range of amplified fragments (bp)	Polymorphic bands	Percentage of polymorphism (%)	No. of Non-parental fragments* in F_1	Parent specific polymorphic bands (bp) in F_1 from	
							CtW2-BL1	*CtB3-SL1*
SCoT -01	CAACAATGGCTACCACCA	5	389-1077	---	---	---	---	---
SCoT -02	CAACAATGGCTACCACCC	7	293-1218	---	---	---	---	---
SCoT-03	CAACAATGGCTACCACCG	12	341-1593	---	---	SCoT-03_{341}	---	---
SCoT -04	CAACAATGGCTACCACCT	4	684-2437	---	---	---	---	---
SCoT -05	CAACAATGGCTACCACGA	3	500-1250	---	---	---	---	---
SCoT -06	CAACAATGGCTACCACGC	8	151-1706	---	---	---	---	---
SCoT -07	CAACAATGGCTACCACGG	6	967-2096	---	---	SCoT-07_{539}	---	---
SCoT-09	CAACAATGGCTACCAGCA	5	394-2260	---	---	---	---	---
SCoT-10	CAACAATGGCTACCAGCC	5	1666-3916	---	---	---	---	---
SCoT-11	AAGCAATGGCTACCACCA	4	509-1255	---	---	---	---	---
SCoT-12	ACGACATGGCGACCAACG	3	366-676	---	---	---	---	---
SCoT-13	ACGACATGGCGACCATCG	2	310-607	---	---	---	---	---
SCoT-14	ACGACATGGCGACCACGC	6	313-1351	---	---	---	---	---
SCoT-15	ACGACATGGCGACCGCGA	2	257-358	---	---	---	---	---
SCoT-16	ACCATGGCTACCACCGAC	5	550-1658	---	---	---	---	---
SCoT-17	ACCATGGCTACCACCGAG	3	590-1546	---	---	---	---	---
SCoT-18	ACCATGGCTACCACCGCC	8	316-1805	---	---	---	---	---
SCoT-19	ACCATGGCTACCACCGGC	14	341-2231	SCoT-19_{583}	7.14	SCoT-19_{548} SCoT-19_{480}	---	583
SCoT-20	ACCATGGCTACCACCGCG	5	500-2210	---	---	---	---	---
SCoT-21	ACGACATGGCGACCCACA	5	231-1023	---	---	---	---	---
SCoT-22	AACCATGGCTACCACCAC	7	269-1132	---	---	---	---	---
SCoT-23	CACCATGGCTACCACCAG	4	275-977	---	---	---	---	---
SCoT-24	CACCATGGCTACCACCAT	6	480-1669	SCoT-24_{562}	16.66	---	---	SCoT-24_{562}
SCoT-25	ACCATGGCTACCACCGGG	8	369-2654	SCoT-25_{1308}	12.5	---	SCoT-25_{1308}	---
SCoT-26	ACCATGGCTACCACCGTC	7	294-963	SCoT-26_{566}	14.28	---	---	SCoT-26_{566}
SCoT-27	ACCATGGCTACCACCGTG	8	527-2386	SCoT-27_{647}	12.5	---	SCoT-27_{647}	---
SCoT-28	CCATGGCTACCACCGCCA	10	474-1552	SCoT-28_{1431} SCoT-28_{981}	20.0	---	---	SCoT-28_{1431} SCoT-28_{981}
SCoT-29	CCATGGCTACCACCGGCC	14	335-2523	---	0	---	---	---
SCoT-30	CCATGGCTACCACCGGCG	8	390-2954	---	12.5	SCoT-30_{438}	---	---
SCoT-31	CCATGGCTACCACCGCCT	11	326-2477	SCoT-31_{774}	9.09	SCoT-31_{326}	SCoT-31_{774}	---
SCoT-32	CCATGGCTACCACCGCAG	9	116-2000	SCoT-32_{1030} SCoT-32_{830}	22.22	---	SCoT-32_{1030} SCoT-32_{830}	---
SCoT-33	CCATGGCTACCACCGCAG	5	210-1000	---	0	---	---	---
SCoT-34	ACCATGGCTACCACCGCA	4	339-1176	---	0	---	---	---
SCoT-35	GCAACAATGGCTACCACC	6	210-2555	SCoT-35 $_{1430}$ SCoT-35_{210}	33.33	---	SCoT-35_{1430} SCoT-35_{210}	---
SCoT-36	GCAACAATGGCTACCACC	5	449-899	SCoT-36_{899}	20.0	---	---	SCoT-36_{899}
	Total	**224**		**13**	**5.80**	**6**	**7**	**6**

CtB3-SL1: 0.104±0.02; *acc. CtW2-BL1:* 0.514±0.019). This variation might be attributed to the genetic recombination favouring conglomeration of suitable alleles, expression of genes producing key enzymes of metabolic pathways and growth environment which probably necessitated the production and accumulation of more taraxerol as reported for different bioactive metabolites in several medicinal species (Amoo and Van Staden 2013).

Mitotic analysis of F_1 hybrid revealed its chromosome count as 2n=16 similar to its parents. Sixteen dis-

tinguished chromosomes were also observed in the mitotic metaphase and they were separated in normal fashion during anaphase. Meiotic analysis revealed formation of eight bivalents at diakinesis and metaphase-I and 8II: 8II separation at anaphase-I, which might be due to homology between the parents. As result the pollen fertility of F_1 hybrid is almost equivalent to its parents. These cytological observations along with morpho-physiological traits could be helpful for the characterization of the F_1 hybrids of *C.* ternatea as reported in many species.

The homologous multigene families control the expression seed protein profile across the species, thus the seed protein marker exhibits monogenic segregation where the presence of polypeptide being completely dominant over absence, and in some cases, co-dominance for molecular weight variants also noticed (Osborn 1988). Mutations or deletions of structural genes coding for these polypeptides or their regulatory loci might lead to lack of expression of the concerned polypeptides (Panigrahi et al. 2007). This kind of variations in seed protein marker profiling led the use this as as reliable markers for verification of hybridity of inter-varietal crosses (Bennet et al. 1991), and inter-specific (Panigrahi et al. 2001, Jisha et al. 2011, Mishra et al. 2012). In the present study SDS-PAGE of seed albumins revealed inheritance of three polymorphic polypeptides (Mw 31.62, 22.38 and 18.81 KDa) specific to pollen parent *C. ternatea acc. CtW2-BL1* in the F_1 hybrid. Since *C. ternatea acc. CtW2-BL1* was used as pollen parent, the appearance of these unique albumin polypeptides in the F_1 hybrid can potentially be used as markers for identification of hybrids involving at least *C. ternatea acc. CtW2-BL1* as pollen parent as reported in *Cajanus cajan* (Panigrahi et al. 2007)

RAPD, SCoT and ISSR marker analysis relies on differential enzymatic amplification of targeted DNA fragments on the basis of primer annealing sequence of the genome. RAPD is being random in nature, this kind of DNA markers were ubiquitously distributed throughout the genome, and capable of detecting a high level of polymorphism. Whereas ISSR is simple sequence repeat specific and SCoT is the specific to the conserved sequence around the initiating codon of the gene. These markers have also been successfully utilized in several crop species for diverse breeding efforts including identification and characterization of the hybrids. In the present study, RAPD, ISSR and SCoT markers showed 17.32, 2.56 and 5.80% polymorphism among the parents. Both ISSR and SCoT analysis showed lower polymorphism (2.56% and 5.80%) in comparison to RAPD markers in the present study. There are some contradictory reports on detection of polymorphism by RAPD and ISSR markers, ISSR markers showed more polymorphism than RAPD markers (Godwin et al. 1997; Lima-Brito et al. 2006; Nagaoka et al. 1997; Zietkiewicz et al. 1994) and vice versa (Muthusamy et al. 2008). This contradiction might be due to the use of different decamer oligonucleotides or SSR motifs as primers, and varied primer-annealing site in the genomes. Again, ISSR and SCoT polymorphism depends on the frequency of SSR motifs and conserved sequence around the initiating codon, respectively (Depeiges et al.1995; Collard and Mackill 2009) which vary within a species or even varieties targeted. Identification of inter and intra-specific hybrids has been carried out in several species using either RAPD or ISSR markers individually, or in combination (Goldmann et al. 2008; Jisha et al.2011; Bianco et al. 2011, Mishra et al. 2012). In this study 22 RAPD, one ISSR and 13 SCoT markers were found to be inherited to the putative F_1 hybrid, and among them 11 RAPD and six SCoT markers, unique to pollen parent (*C. ternatea*, *acc. CtW2-BL1*), were very vivid in its appearance for the identification of F_1 hybrid. In the present study, several non-parental fragments have also been amplified in the F_1 hybrid, and this might be due to either DNA recombination followed by minor genomic reorganization during the hybridization (Huchett et al. 1995), or loss of priming sites due to chromosomal crossing over during meiosis (Smith et al.1996). As the objectives is to identify the hybrids and to confirm the hybrid nature of putative seedlings at the juvenile stage, screening of the putative F_1 hybrids using pollen parent-specific RAPD, ISSR and SCoT markers contribute economic significance to this medicinal plant. The findings from the present study, it has been assorted that use of seed protein profiling and DNA marker analysis complements the characterization of intra-specific F_1 hybrid along with morpho-physiological traits and cytogenetic analyses more precisely. Further, these inherited seed albumin and DNA markers could also be used for further studies in gene mapping, marker-assisted breeding involving intra-specific hybridization in *C. ternatea* aiming at enhanced metabolite content of therapeutic importance.

ACKNOWLEDGEMENTS

The authors are highly thankful to DST, Govt. of Odisha for providing Biju Patnaik Research Fellowship to the author (AN) for pursuing the doctoral degree, and acknowledge the laboratory facility provided by the Vice Chancellor, Sambalpur University.

FUNDING

Research work funded by Department of Science and Technology, Govt. of Odisha and UGC, Govt. of India.

REFERENCES

Ali Z, Ganie S, Narulaa A, Sharma M, Srivastava P. 2013. Intra-specific genetic diversity and chemical profiling of different accessions of *Clitoria ternatea* L. Industrial Crops Prod. 43: 768-773

Amoo SO, Van Staden J. 2013. Influence of plant growth regulators on shoot proliferation and secondary metabolite production in micropropagated *Huernia hystrix*. Plant Cell Tissue Organ Cult. 112: 249-256

Anonymous.1962. The Wealth of India. Council of Scientific and Industrial Research, New Delhi.

Behera M, Mishra RR, Bindhani B, Panigrahi J (2010) Cytological analysis in *Asteracantha longifolia* L. (NEES)-a medicinal herb. Int J Bot. 6: 132-135

Bennet M, Sajid GM, Chattern NJ, Asaif KH.1991. Electrophoretic characterization of quack grass and blue bunch wheat grass hybrid seeds. Seed Sci Tech.19: 355–362.

Bianco C, Fernandez J, Migiliaro D, Crino P, Egea-Gilabert C. 2011. Identification of F_1 hybrids of artichoke by ISSR markers and morphological analysis. Mol Breed. 27: 157-170.

Bishoyi A, Pillai V, Geetha K, Maiti S. 2014. Assessment of genetic diversity in *Clitoria ternatea* L. populations from different parts of India by RAPD and ISSR markers. Genet Res Crop Evol. 61: 1597-1609.

Braig M, Lee S, Loddenkemper C, Rudolph C, Peters AH, Schlegelberger B, Stein H, Dorken B, Jenuwein T, Schmitt CA. 2005. Oncogene-induced senescence as an initial barrier in lymphoma development. Nature. 436: 660–665.

Chandra A. 2011. Use of EST database markers from *Medicago truncatula* in the transferability to other forage legumes. J Environ Biol. 32: 347-354.

Chen C, Kong ANT. 2005. Dietary cancer-chemopreventive compounds: from signaling and gene expression to pharmacological effects. Trends Pharmacol Sci. 26: 318–326.

Collard B, Mackill D. 2009. Start Codon Targeted (SCoT) Polymorphism: A Simple, Novel DNA Marker Technique for Generating Gene-Targeted Markers in Plants, Plant Mol Biol Rep. 27: 86–93.

Cook BG, Pengelly BC, Brown SD, Donnelly JL, Eagles DA, Franco MA, Hanson J, Mullen BF, Partridge IJ, Peters M, Schultze-Kraft R. 2005.Tropical forages: an interactive tool [CD-ROM]. CSIRO, DPI&F(Qld), CIAT and ILRI, Brisbane, Australia

Depeiges A, Goubely C, Lenoir A, Cocherels S, Picard G, Raynal M, Grellet F, Delseny M. 1995. Identification of the most represented repeated motifs in Arabidopsis thaliana microsatellite loci. Theor Appl Genet. 91: 160-168.

Fukui Y, Tanaka Y, Kusumi T, Iwashita T, Nomoto K (2003) A rationale for the shift in colour towards blue in transgenic carnation flowers expressing the flavonoid 3', 5'hydroxylase gene. Phytochemistry 63: 15-23.

Ganie S, Srivastava P, Narula A, Ali Z, Sharma M. 2012. Authentication of shankhpusphi by RAPD markers. Eurasia J Biosci. 6: 39-40.

Godwin ID, Aitken EA, Smith LW. 1997. Application of inter simple sequence repeat (ISSR) markers to plant genetics. Electrophoresis. 18: 1524-1528.

Goldmann JJ.2008. The use of ISSR markers to identify Texas blue grass interspecific hybrids. Plant Breed.127: 644-646.

Hemalatha T, Shanmugasundaram P. 2010. Efficiency of DNA marker system in discriminating *Cajanus cajan* (L.) Millsp. and its wild relatives. Indian J Plant Genet Resour. 23: 93-99.

Huchett BI, Botha FC. 1995. Stability and potential use of RAPD markers in sugarcane genealogy. Euphytica. 86: 117–125.

ILDIS.1994. Plants and their constituents. In: Bisby FA (ed) Phytochemical dictionary of the Leguminosae. Chapman and Hall, New York, pp. 1–748.

Jisha MS, Beevy SS, Nair GM. 2011. Species relationship in the genus CucumisL. revealed by crossability, chromosome pairing and seed protein electrophoresis. Nucleus. 54: 35-38.

Joson MT, Ramirez DA.1991. Cytology of *Clitoria ternatea* L. Philipp Agriculturist 74: 121-132.

Kapoor LD. 2005. Handbook of Ayurvedic Medicinal Plants. CRC press, Boca Raton, London.

Katsumoto Y, Fukuchi-Mizutani M, Fukui Y et al. 2007. Engineering of rose flavonoid biosynthetic pathway successfully generated blue hued flowers accumulating Delphinidin.

Khare CP. 2004. Encyclopedia of Indian Medicinal Plants. Springer Publication, Heidelberg.

Kumar V, Mukherjee K, Kumar S, Mal M, Mukherjee PK. 2008. Validation of HPTLC Method for the analysis of Taraxerol in *Clitoria ternatea*. Phytochem. Anal. 19: 244-250.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685.

Lima-Brito J, Carvalho A, Martin A, Heslop-Harrison JS, Guedes-Pinto H.2006. Morphological, yield, cytological and molecular characterization of a breed wheat × tritorderm F_1 hybrid. J Genet. 85: 123-131.

Margret AA, Begum TN, Parthasarathy S, Suvaithenamudhan S. 2015. A Strategy to employ *Clitoria ternatea* as prospective brain drug confronting monamine oxidase (MAO) against neurodegenerative diseases and depression. Nat Prod Biosprospect 5: 293-306.

Mishra RR, Sahu A, Rath SC, Mishra SP, Panigrahi J. 2012. Cyto-morphological and molecular characteriza tion of *Cajanus cajan* × *C. scarabaeoides* F_1 hybrid. The Nucleus. 55: 27-35.

Mishra SK, Panigrahi J. 2017. Identification of interspecific hybrid between *Cajanus cajan* (L.) and *C. cajanifolius* (H.) using cyto-morphological and DNA markers. Electronic J Plant Breed. 8(1): 63-71.

Mohanty JB, Naik BS, Panigrahi J, Kole C .2001.Identification of mungbean cultivars based on electrophoretic patterns of seed protein. Crop Res .21: 134-138.

Mukherjee PK, Kumar V, Kumar NS, Heinrich M. 2008. The ayurvedic medicine *Clitoria ternatea* – from traditional use to scientific assessment. J. Ethnopharmacol. 120, 291-301.

Muthusamy S, Kanagarajan S, Ponnusamy S. 2008. Efficiency of RAPD and ISSR marker system in accessing genetic variation of rice bean (*Vigna umbellata*) land races. Electronic J Biotechnol. 11: 1-10.

Nagaoka T, Ogihara Y. 1997. Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP and RAPD markers. Theor Appl Genet. 94: 597–602.

Niering P, Michels G, Watjen W, Ohler S, Steffan B, Chovolou Y, Kampkotter A, Proksch P, Kahl R. 2005. Protective and detrimental effects of kaempferol in rat H4IIE cells: implication of oxidative stress and apoptosis. Toxicol Appl Pharmacol. 209: 114–122.

National Plant Germplasm System. 2008. Germplasm resources information network (GRIN). Database Management Unit (DBMU), National Plant Germplasm System, U.S. Department of Agriculture, Beltsville

Osborn TC. 1988. Genetic control of bean seed protein. CRC Crit Rev Plant Sci. 7: 93-116.

Panigrahi J, Patnaik SN, Kole C. 2001. Identification of hybrids of pigeonpea (*Cajanus cajan*) and *C. cajanifolius* using male specific protein markers. Indian J Genet Plant Breed. 61: 367-368.

Panigrahi J, Kumar DR, Mishra M, Mishra RP, Jena P. 2007. Genomic relationships in the genus *Cajanus* as revealed by seed protein (albumin and globulin) polymorphisms. Plant Biotechnol Rep. 1: 109-116.

Parrotta JA.2001.Healing Plants of Peninsular India. CABI Publishers, New York, USA.

Prajapati ND, Purohit SS, Sharma AK, Kumar T. 2003. A Handbook of Medicinal Plants A Complete Source Book. Agrobios Publication, Jodhpur, India.

Sethiya NK, Nahata A, Mishra SH, Dixit VK.2009. An update on Shankhpushpi, a cognition-boosting ayurvedic medicine. J Complimentary Integr Med. 7: 1001-1022.

Singletary KW, Jung KJ, Giusti M . 2007. Anthocyanin-rich grape extract blocks breast cell DNA damage. J Med Food 10: 244-251.

Sivaramakrishnan S, Seetha K, Rao AN, Singh L. 1997. RFLP analysis of cytoplasmic male sterile line of pigeonpea (*Cajanus cajan* L. Millsp.) developed by interspecific crosses. Euphytica. 93: 307-312.

Smith JF, Burke CC, Wangner WL. 1996. Interspecific hybridization in natural population of Cyrtandra (Gesneriaceae) on the Hawaian islands: evidence from RAPD markers. Plant Sys Evol. 200: 61-77.

Swain S, Rout K, Chand P.2 012. Production of Triterpenoid Anti-cancer Compound Taraxerolin Agrobacterium-Transformed Root Cultures of Butterfly Pea (*Clitoria ternatea* L.). Appl Biochem Biotechnol. 168: 487-503.

Swati DY, Malode SN,Waghmare VN and Thakre P.2011. Genetic relationship and diversity analysis of *Clitoria ternatea* variants and *Clitoria biflora* using Random amplified polymorphic DNA (RAPD) markers. Afr J Biotechnol. 10: 18065-18075

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

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

Citation: K. Gautam, R. Raina (2019) Floral architecture, breeding system, seed biology and chromosomal studies in endangered Himalayan *Angelica glauca* Edgew. (Apiaceae). *Caryologia* 72(3): 23-34. doi: 10.13128/caryologia-755

Published: December 13, 2019

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

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

Floral architecture, breeding system, seed biology and chromosomal studies in endangered Himalayan *Angelica glauca* Edgew. (Apiaceae)

Kamini Gautam[1,2,*], Ravinder Raina[1,3]

[1] *Dr. YSP University of Horticulture and Forestry, Solan, Himachal Pradesh, India*
[2] *Grassland and Silvipasture Management Division, ICAR-Indian Grassland and Fodder Research Institute, Jhansi, Uttar Pradesh, India*
[3] *Amity Food and Agriculture Foundation, Amity University, Noida, Uttar Pradesh, India*
*Correspondence author: kaminigautam1989@gmail.com

Abstract. Endangered *Angelica glauca* an important medicinal plant of temperate Himalaya is valued for its roots which are used to treat several diseases besides food flavouring. Reproductive biology studies conducted in this species for the first time have revealed i). presence of umbels of different orders with only bisexual flowers ii). occurrence of sterile seeds (without embryo) apart from fertile ones iii). seed set in only early blooming umbels (primary and lateral-I) iv). 2n=22 chromosomes besides presence of chromosomes in a group at metaphase and anaphase-I and cytomixis in some pollen mother cells and v). extreme protoandry and cross pollination behavior (upto 95%) of the species. These observations have implications for developing any conservation plan for the species.

Keywords. Endangered, umbel order, Apiaceae, cross pollination, low seed set, embryo less seeds.

INTRODUCTION

Apiaceae an angiosperm family consists of 300-455 genera and 3000-3750 species worldwide (Pimenov and Leonov 2004) and many of these species are highly valued for being economically and medicinally important (Butola and Badola 2006; Sher et al. 2011). The genus *Angelica* is one of the very important genera belonging to family Apiaceae and is represented by about 110-115 species worldwide and almost 87 species in Asia (Pimenov and Leonov 2004). This genus is represented by mainly three species viz. *A. glauca* Edgew., *A. archangelica* L. and *A. nubigena* Clarke in Himalayan region. *A. nubigena* is poorly known species found in Sikkim (Pimenov and Kljuykov 2003) and other species i.e. *A. cyclocarpa* (C.Norman) M. Hiroe and *A. oreadum* Diels have also been reported from Indian Himalaya, Pakistan and Afghanistan (Pimenov and Kljuykov 2003).

Angelica glauca Edgew. (Family: Apiaceae; English name: Himalayan Angelica; Local name: chora, chokhara & gandrayan) is an endangered perennial temperate medicinal and aromatic herb distributed in moist and shady regions of Himalaya at an altitude of 2000-3800m amsl (IUCN 1993; Samant et al. 1998; Chauhan 1999; Butola and Budola 2004; Samant et al.2009) in Afganistan, Pakistan and India (Jammu and Kashmir, Himachal Pradesh and Uttrakhand) (Bisht et al. 2003; Butola and Budola 2004; Saeed and Sabir 2008; Butola and Vashistha 2013).Valued for roots which are used to treat dismenhorrea, metorrhagia, amenhorrea, polycystic ovary syndrome, rheumatism, infantile atrophe (Bisht et al. 2003; Butola and Samant 2006; Butola and Budola 2008; Butola and Vashistha 2013; Goswami et al. 2012), also acts as stimulant, cholagogue, cardio-active, carminative, sudorfic and expectorant (CSIR 1985; Singh and Rawat 2000; Bisht et al. 2003; Butola and Samant 2006). Besides this, roots also yield essential oil used to flavour liquor and food items (Nautiyal and Nautiyal 2004; Butola and Samant 2006). Due to remote distribution of *A. glauca* in inaccessible areas of Himalaya coupled with small size of flower because of being an Apiaceae member, very less work has been carried out on its reproductive biology. However, breeding behavior and reproductive biology studies are crucial for understanding plant pollinator interaction, reproductive bottlenecks as well as for developing conservation plan. Therefore reproductive biology has been studied in details for the first time in this species under the present investigation.

Material and methods

Studies were carried out at Shillaru (2130m amsl, 30°45'00.48''N, 76°59'12.22''E; District Shimla, Himachal Pradesh, India); at Shilly (1550m amsl; 30°54'30''N, 77° 07'30''E; District Solan, Himachal Pradesh, India) and Medicinal plants laboratory of department of Forest Products, College of Forestry, Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan-173230 (Himachal Pradesh, India) during years 2013-2016. Population Shillaru had almost 100 plants whereas in Shilly population 50 plants were present.

The vegetative and floral studies were conducted as per standard literature (Lawrence 1951; Weberling 1989; Kaufman et al. 1989). Pollen-ovule ratio was studied as per Cruden (1977) and pollen viability was calculated on the basis of one percent acetocarmine staining test.

Young floral buds, fixed in absolute alcohol, glacial acetic acid and chloroform in the ratio of 1:1:1(v:v:v) for 24 hours, washed and then stored in 70% alcohol at low temperature, were used for meiotic studies. One percent acetocarmine stain was used for chromosomal staining by usual squash method. For open pollination plants with unopened healthy umbels were tagged and left as such, whereas for assessing autogamy umbels were enclosed (Figure 2a) at pre flowering stage. Increase in ovary size and its transformation into fruit was taken as the basis of fruit set. Seed viability was tested by Topographical Tetrazolium Test (TTZ) test and germination by petri plate method.

Petri-plate germination test was performed at 23 ± 2°C in growth chamber and radicle protuberances were taken as sign of germination. Topographical Tetrazolium Test (TTZ) (0.1% pH 6.0) for 48 hours after extracting seeds was conducted by soaking seeds in water, then excised to expose embryo followed by immersing in TTZ solution (0.1% pH 6.0) under dark conditions for 48 hours. Darkly red stained embryos were taken as viable.

Statistical analysis was made as per CRD factorial (seed germination and viability testing under laboratory conditions), RBD factorial (seed set % in field) as well as T-test (pollination studies). Statistical analysis was conducted as per Gomez and Gomez (1984). Ocular and stage micrometer (ERMA, Tokyo, Japan) were used for micro-measurements and microscopic examination was made using Olympus trinocular research microscope (Model - CH20iBIMF, New Delhi, India).

RESULTS

Morphology and floral architecture

The qualitative and quantitative features are tabulated (Table 1). Plants of this species are erect perennial herbs, inflorescence is compound umbel with umbels of different order i.e. primary, lateral-I, lateral-II and lateral -III umbels based on whether they are borne on main stem or lateral branches (Figure1a, b). Percentage of plants with different umbel order varied in two studied populations (Table 2) and quantitative features of umbels are presented in Table 3. All the morphological features of stem, roots, leaves, inflorescence, flower, fruit and seeds were similar to earlier reports except for the presence of variation in seed size and presence of seeds without embryo which are is being reported for the first time in *A. glauca* (Figure 2b, c).

Phenology

Sprouting starts in spring season (last week of April month onwards) and continues up to June month (first week). Floral buds start appearing during July first week

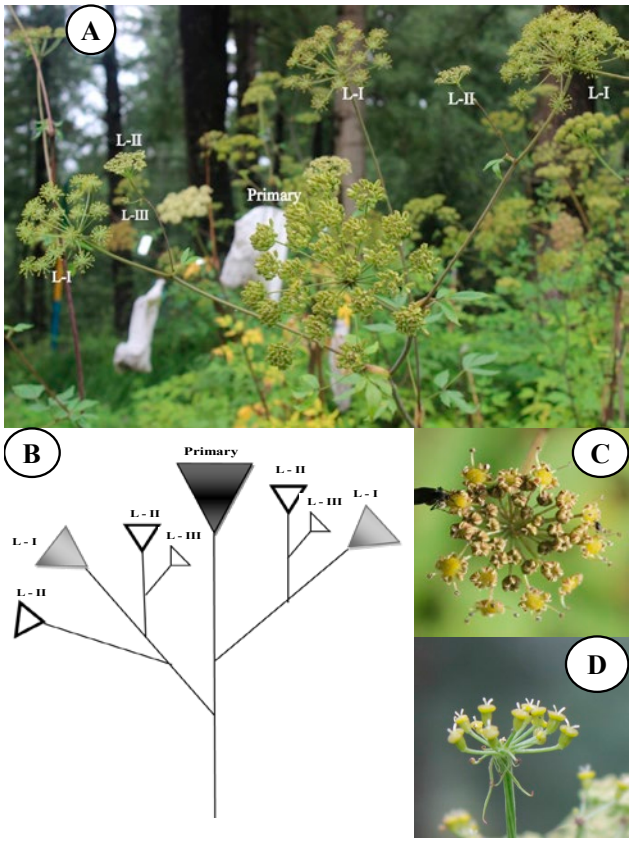


Fig. 1. *A. glauca* a. Arrangement of umbel orders *primary, lateral-I (L-I), lateral –II (L-II), lateral –III (L-III)*; b. Schematic representation of a; c. Anther dehiscence stage; d. Protruding style at receptive stage.

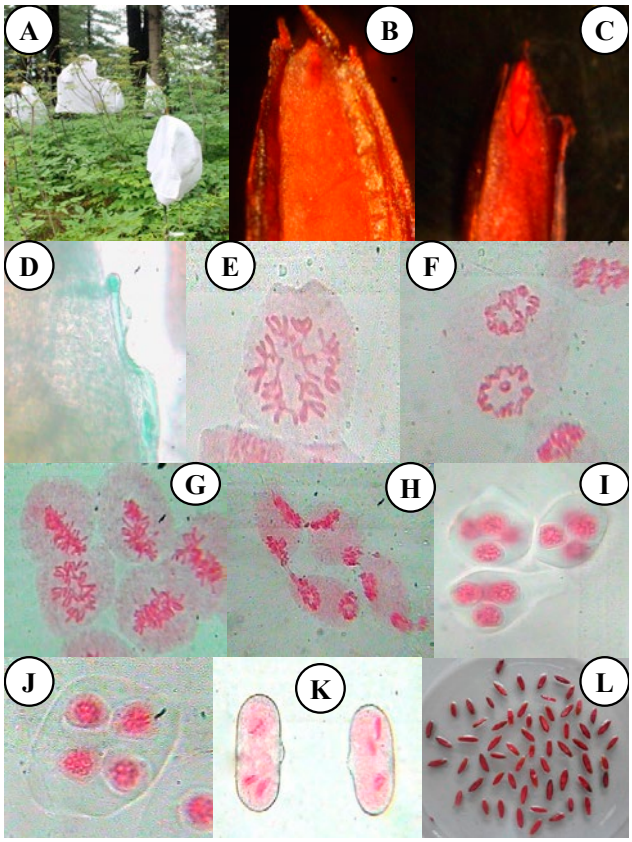


Fig. 2. *A. glauca* a. Bagging for autogamy; b & c. Seed without and with embryo respectively after TTZ staining; d. Pollen germination on receptive stigma; e. Metaphase-I (n=11, 2n=22); f. Ring formation at poles at anaphase-I; g. Clumping of chromosomes at Metaphase-I; h. Pollen mother cells showing cytomixis i. Decussate and tetrahedral tetrads; j. Isobilateral tetrads; k. Trinucleate pollen grains; l. Seed viability through TTZ test.

to mid of August month. Primary umbel floral buds appear first followed by buds of lateral-I, lateral-II and lateral-III umbels. Peak flowering occurs during August and is asynchronous even among plants occurring at same niche. Within a plant also, phenological events are asynchronous among primary, lateral-I and lateral-II umbels. Fruit formation commence during the last week of August completing (full maturity) by September last week. Fruit shedding occurs with beginning of October month onwards and physical as well as physiological changes leading to the prennation commence with the beginning of autumn season. This inactive phase lasts upto next spring season.

Breeding system studies

Floral biology

Anther dehiscence (Figure 1c) asynchronously starts with the opening of floral buds through longitudinal slits and continues for 2-3 days. Stigma become receptive (observed by *in vivo* artificial pollination and resultant pollen germination) when all the anthers of a flower are shed and style reached its maximum length after protruding out of stylopodium (Figure 1d). At stigma receptive stage, stylopodium is with shiny surface. Period of stigma receptivity depended upon umbel order.

Elongated, trinucleate (at shedding stage) and bicolpate pollen show 76% to 100% (average 92.64 ± 1.71%) stainability and their number/flower vary from 17400 to 40700 (average 26128.75± 1323.41). With two ovules/flower, pollen-ovule ratio ranged from 8700 to 20350 (average 13064.37± 661.71). Number of seeds produced ranged from 334 to 1024 (average 670.27 ± 65.24) in primary umbel and 112 to 344 (average 216.64 ± 18.88) in lateral-I umbel whereas, no seed set in lateral-II and lateral-III umbels was observed as these umbels dry before fruit formation.

Table 1. Qualitative and quantitative morphological features of *A. glauca*.

Plant part	Qualitative	Quantitative
Habit and habitat	Erect perennial temperate and alpine herb.	
Stem	Erect, cylindrical, hollow inside, smooth and swollen at nodes. Covered with white powder and variously colored (entire shoot purple; purple upto middle and green at top or opposite; to green with purple patches).	**L:** 172.67 ± 6.36 cm
Roots	Perennial consisting of tuberous roots, pale yellow to yellowish brown, surface smooth or wrinkled and occasionally tap root splits into two near collar region.	**Tap L:** 18.90 ± 1.66 cm **B:** 13.56 ± 0.97 mm **Secondary L:**12.53 ± 1.23 cm **B:** 1.40 ± 0.36 mm
Leaves	Large, petiolated, tripinnate, alternate, with very long rachis. Petiole base sheathing. Leaflets: lance-ovate to ovate, tip narrowly-acute to acute, base cuneate, margin irregularly toothed and reticulate venation. Adaxial surface of leaflets dark green and smooth. Abaxial surface grayish white and smooth.	**Cauline leaves/ plant:** 5-12
Inflorescence	Compound umbel with umbels of different orders.	**Umbels/plant:** 2 –9
Involucre bracts	6-10 in number, linear and green colored.	**L:** 2.73 ± 0.27 cm
Bracts	4-11 in number, linear and green colored.	**L:** 1.72 ± 0.17 cm
Flower	Bisexual, pedicillate, epigynous, actinomorphic and pentamerous.	**Spread:** 3.39 ± 0.08 mm
Pedicel	Green colored, length decrease from peripheral towards the centre.	
Calyx	Absent or obsolete.	
Corolla	Petals five, free, valvate, obovate with inward curved tip, green in bud stage and whiter on maturity.	**L:** 2.08 ± 0.03 mm **B:** 1.52 ± 0.07 mm
Androecium	Stamens five, green colored, bilobed, dorsifixed, exerted, alternate to petals, dehisce by longitudinal slits and filaments green colored. Anthers remain bend inwards in bud stage and spread outwards at maturity.	**Filament L:** 3.15 ± 0.09 mm **Anther lobe L:** 0.97 ± 0.02 mm **Anther lobe B:** 0.77 ± 0.01 mm
Gynoecium	Ovary inferior, bicarpillary syncarpous, bilocular bearing single solitary ovule in each locule and placentation apical. Style bifid, erect, white coloured and attain full development after anther dehiscence. Stylar base swollen to form stylopodium.	**Ovary L:** 1.30 ± 0.07 mm **B:** 1.72 ± 0.06 mm **Style L:**1.67 ± 0.06 mm **Ovule size:** 0.65 ± 0.04 × 0.29 ± 0.01 mm
Fruit	Fruit mericarp, green colored, oblong, smooth, flat, pale white to brown, on maturity divides longitudinally into two halves joined with the help of carpophores bearing a single seed in each half.	**L:** 1.66 ± 0.05 cm
Seed	Flat, pale whitish to brown, with five ridges, two lateral ridges form oblong membranous wings that surrounds the seed, wing color pale white or brown.	**Small seed L:** 0.59 ± 0.03 cm **Medium seed L:** 0.94 ± 0.02 cm **Large seed L:** 1.34 ± 0.04 cm
Floral formula	♂ , ⊕, $K_{0 \text{ or obsolete}}$, C_5, A_5, $G_{(2)}$	

L: length; B: breadth.

Chromosomal studies

In most of the pollen mother cells, the bivalent upto metaphase stage appeared to be clumped together without clear separation (Figure 2g), however, some cells with separate 11 bivalents were also observed (Figure 2e). Anaphase-I was interesting as chromosomes at each pole were present in groups forming ring like structure and the number of chromosome in each group at each pole was 11 (Figure 2f). In 7-8% pollen mother cells, cytomixis (Figure 2h) was observed, however other abnormalities like laggards, bridges, etc. were absent. Pollen grains were trinucleate at pollen shedding stage (Figure 2k).

Breeding system

Floral visitors like bees, flies, beetles, butterflies and ants were observed visiting its flowers. Open pollination resulted in 670.27 ± 65.24 seeds/primary umbel and under autogamous conditions, only 21.82 ± 15.36 seeds/primary umbel were set (Table 4). Based on the average number of flowers per primary umbels (591 ± 38.96) with two ovules per flower, 56.71 ± 5.52% seed set under open pollination conditions and 2.63 ± 1.82% under autogamous conditions was observed. Out of is 56.71 ± 5.52% seed set under open conditions, after microscopic examinations, 27.49 ± 2.67% of such seeds was with embryo with the rest (29.22 ± 2.84%) being with-

Table 2. Percentage of plants with different umbel order in *A. glauca*:

Population	Primary	Primary + Lateral- I	Primary + Lateral -I + Lateral -II	Primary + Lateral -I + Lateral -II+ Lateral- III
Shillaru	100%	24%	72%	4%
Shilly	100%	60%	40%	0 %

Table 3. Quantitative features of umbels of different order in *A. glauca*:

Umbel order	Characters			
	Primary	Lateral-I	Lateral-II	Lateral-III
Number per plant	1	2-4	0-5	0-1
Diameter	15.56 ± 0.52 cm × 15.6 ± 0.53 cm	11.59 ± 0.80 × 11.53 ± 0.81 cm	3.93 ± 0.21 cm × 3.93 ± 0.21 cm	It was observed to be simple umbel, with upto 10 flowers, very weak and dried later on before blooming.
Umbelet number	18.50 ± 0.86	18.05 ± 0.85	15.08 ± 1.22	
Number of flowers	591 ± 38.96	539.35 ± 16.92	247.47 ± 31.09	
Diameter of peripheral umbelet	3.05 ± 0.07 cm × 3.05 ± 0.07 cm	2.32 cm ± 0.13 × 2.28 ± 0.14 cm	0.75 ± 0.08 cm × 0.75 ± 0.08 cm	
Diameter of central umbelet	2.43 ± 0.06 cm × 2.42 ± 0.06 cm	1.61 ± 0.16 cm × 1.61 ± 0.16 cm	0.43 ± 0.03 cm × 0.43 ± 0.03 cm	
Number of flowers in peripheral umbelets	33.65 ± 0.91	30.95 ± 1.01	16.05 ± 1.15	
Number of flowers in central umbelets	24.90 ± 0.91	20.8 ±0.89	10.9 ± 1.03 cm	
Length of peripheral rays	8.2 ± 0.45 cm	5 ± 0.29 cm	1.17 ± 0.80 cm	
Length of central rays	5.2 ± 0.35 cm	3.01 ± 0.26 cm	0.64 ± 0.05 cm	
Length of flower stalk in peripheral flowers of peripheral umbelets	1.09 ± 0.07 cm	0.85 ± 0.04 cm	0.25 ± 0.02 cm	
Length of flower stalk in central flowers of peripheral umbelets	0.48 ± 0.04 cm	0.32 ± 0.03 cm	0.16 ± 0.02 cm	
Length of flower stalk in peripheral flowers of central umbelets	0.73 ± 0.04 cm	0.54 ± 0.03 cm	0.1 cm	
Length of flower stalk in central flowers of central umbelets	0.36± 0.03 cm	0.19± 0.02 cm	Central flowers were underdeveloped	

Table 4. Impact of different pollination methods on seed set and viability in primary umbel of *A. glauca*:

Pollination Conditions	Observations – Primary umbel						
	Average number of seeds** per umbel*	Total seed** set* %	Seed set % (with embryo)*	Seed set % (without embryo)*	Seed viability %***		100 Seed# weight grams*
					With embryo	Without embryo	
Open Pollination	670.27 ± 65.24	56.71 ± 5.52	27.49 ± 2.67	29.22 ± 2.84	100	0.00	1.20 ± 0.44 g
Self Pollination	21.82 ± 15.36	2.63 ± 1.82	1.27 ± 0.88	1.36 ± 0.93	100	0.00	0.85 ± 0.06 g
T calculated value	9.22	8.87	8.87	8.87			4.19

* Statistically significant.
**On the basis of number of ovules involved in study. Refers to all these structures that appeared to be like seed (with or without embryo).
*** Refers to seeds with embryo only. Seeds without embryo did not show any positive viability due to absence of embryo.
# Refers to all these structures that appeared to be like seed (with or without embryo).

Table 5. Different seed size classes in A. glauca.

Size class	Size of seeds (cm)	100 seed weight in grams (g)
Small	0.59 ± 0.03 (0.4-0.7)	0.72 ± 0.03 g
Medium	0.94 ± 0.02 (0.8-1.0)	1.02 ± 0.04 g
Large	1.34 ± 0.04 (1.1-1.6)	1.18 ± 0.07 g

out embryo. Similarly out of the 2.63 ± 1.82% seed set under autogamous conditions, 1.27 ± 0.88% seed was with embryo with the rest 1.36 ± 0.93% without embryo respectively. 100 seed test weight under open pollination (1.20 ± 0.44 g) was statistically higher to 0.85 ± 0.06 g under autogamous pollination. TTZ test revealed 100% seed viability (Figure 2l) in seeds with embryo in both open as well as autogamous conditions and on the contrary none of the seed without embryo was found to be viable.

Seed biology

Seed size

Differences in seed size were noticed and were categorized into i). small, ii). medium and iii). large seeds (Table 5).

Seed set percentage in different umbel orders of *A. glauca*

670.27 ± 65.24 seeds were obtained in primary umbel which was statistically higher than 216.64 ± 18.88 obtained in lateral-I umbels thus, seed set percentage was 56.71 ± 5.52% in primary and 20.08 ± 1.75% in lateral-I umbel. Out of these only 27.49 ± 2.67% and 6.53 ± 0.57% with seeds with embryo were present in primary and lateral-I umbel respectively which was statistically significant. 100% viability in seed with embryo was observed irrespective of umbel order by TTZ test. Seed set by primary umbel had statistically significant 100 test seed weight (1.20± 0.44 g) as compared to 0.94 ± 0.28 g in lateral-I umbel (Table 6).

Seeds (with embryo) set percentage as influenced by location, umbel order and position within umbel

In Shillaru population (2130 m amsl, district Shimla, HP, India), statistically non-significant difference in percentage of seed with embryo among primary and lateral-I umbels as well as among peripheral and central regions of these umbel orders was observed (Table 7). 53.80% (maximum) seeds with embryo were observed in peripheral regions of primary umbels and 32.38% (minimum) in central region of lateral-I umbels which was however statistically non significant. On overall basis, 48.47% seeds with embryo were obtained in primary and 32.52% in lateral-I umbel (Table 7).

In Shilly population (1550 m amsl, district Solan, HP, India), maximum (66.18%) seeds with embryo were obtained in central region of primary umbel and minimum (36.68%) in central region of lateral-I umbel which was however statistically non-significant (Table 7). On overall basis maximum (56.56%) seeds with embryo were obtained in primary umbel which was statistically higher to minimum (40.11%) obtained in lateral-I umbels. Maximum (51.43%) seeds with embryo were obtained in central region of umbels and minimum (45.24%) in peripheral regions of umbels which was, however statistically non-significant (Table 7).Amongst the two populations, on overall basis 48.34% (Shilly) and 40.49% (Shillaru) seeds with embryo were obtained which was statistically non-significant (Table 8).

Table 6. Seed set percentage in different umbels of *A. glauca*:

Umbels	Observations						
	Average number of seed per umbel*#	Total seed set*# %	Seed set % (with embryo)*	Seed set % (without embryo)*	Seed viability %**		100 Seed weight*** grams*
					With embryo	Without embryo	
Primary Umbel	670.27 ± 65.24	56.71 ± 5.52	27.49 ± 2.67	29.22 ± 2.84	100	0.00	1.20 ± 0.44 g
Lateral-I Umbel	216.64 ± 18.88	20.08 ± 1.75	6.53 ± 0.57	13.55 ± 1.18	100	0.00	0.94 ± 0.28 g
T calculated value	6.37	6.03	7.30	4.85			4.37

* Statistically significant
# On the basis of number of ovules involved in study. Refers to all these structures that appeared to be like seed (with or without embryo).
**Refers to seeds with embryo only. Seeds without embryo did not show any positive viability due to absence of embryo.
*** Refers to all these structures that appeared to be like seed (with or without embryo).

Table 7. Percentage of seeds with embryo among different umbel order vis-à-vis umbel part in population in *A. glauca*.

Umbel order	Umbel part					
	Shillaru population Seed set %			Shilly population Seed set %		
	Peripheral	Central	Mean	Peripheral	Central	Mean
Primary	53.80 (47.13)	43.13 (39.15)	48.47 (43.14)	46.94 (46.21)	66.18 (58.12)	56.56 (52.16)
Lateral-I	32.65 (34.36)	32.38 (32.37)	32.52 (33.36)	43.55 (41.17)	36.68 (37.12)	40.11 (39.15)
Mean	42.23 (40.74)	37.76 (35.76)		45.24 (43.69)	51.43 (47.62)	
$Cd_{0.05}$						
Umbel order		NS*			12.13	
Within umbel		NS			NS	
Umbel order X within umbel		NS			NS	

Values in parentheses are Arc Sine transformed values.
* Non significant.

Seed size vis-à-vis percentage of seeds with embryo

The large sized seed consisted of 52.12% seeds with embryo at Shillaru as against 58.12% obtained at Shilly, (statistically non significant). Amongst the small seeds, only 37.67% (Shillaru) and 39.15% (Shilly) seeds were with embryo (Table 9). Amongst medium sized seeds 28.66% (Shillaru) and 41.66% (Shilly) were with embryo (table 9). There was observed no statistically significant difference amongst the two locations i.e. Shilly and Shillaru but significant difference in percentage of seed with embryo amongst seeds of different size class was observed at both locations (Table 9) with large seeds having higher proportion of seeds with embryo (55.12%).

Seed germination

Seed size class wise, inter and intra population seed germination was conducted and Shillaru (2130 m amsl, district Shimla, HP, India) population gave maximum (31.00%) germination which was statistically higher (Table 10). Seeds of Kilba (3200 m amsl, 31°31' 18.17" N; 78°11' 49.30"E district Kinnaur, HP, India) population did not germinate at all and in case of Khan Jungle (2300 m amsl, 30°49' 13.40" N; 77°27' 47.82" E, district Sirmour, HP, India) population, large sized seeds gave maximum germination (24.00%) and medium seeds gave minimum germination (6.00%) (Table 10). In case of Jagatsukh (1982 m amsl, 32° 11' 43.20" N; 77° 12' 31.82" E, district Kullu, HP, India) population large seeds gave maximum germination (8.00%) and small seeds did not germinate at all (Table 10). In case of Thandi Dhar (2240 m amsl, 30° 54' 51.42"N; 77° 24' 44.45"E, district Sirmour, HP, India) population, medium seeds gave maximum germination (26.67%) and large seeds gave minimum germination (8.33%) (Table 10). In case of seeds from Rohru Forest Division (2700 m amsl, 31°07'09.49"N; 77°37'35.45"E, district Shimla, HP, India), small seeds gave maximum germination (34.00%) and large seeds minimum (13.33%) (Table 10). In case of Shillaru population, medium seeds gave maximum germination (48.00%) and large seeds gave minimum germination (10.00%) (Table 10). On overall basis, non significant impact of seed size on seed germination was observed (Table 11).

Table 8. Overall percentage of seeds (with embryo) comparison between two population of *A. glauca.*

Populations	Seed with embryo (%)
Shillaru	40.49%
Shilly	48.34%
T calculated value	1.37*

* Non significant.

Table 9. Percentage of seed with embryo amongst different seed size classes in *A. glauca.*

Population	Seed size			
	Small	Medium	Large	Mean
Shillaru	37.67 (34.96)	28.66(31.64)	52.12(46.34)	39.49(37.65)
Shilly	39.15(38.42)	41.66(39.90)	58.12(54.95)	46.31(44.42)
Mean	38.41 (36.69)	35.16(35.77)	55.12 (50.65)	
$Cd_{0.05}$				
Sites		NS*		
Seed size		9.69		
Site X size		NS		

* Non significant.
Values in parentheses are Arc Sine transformed values.

Table 10. Site wise open pollination set seed germination response vis-à-vis seed size in *A. glauca*.

Sites	Category			
	Small	Medium	Large	Mean
Khan Jungle	20.00 (26.54)	6.00 (14.12)	24.00 (29.30)	16.67 (23.32)
Kilba	-	0.00 (0.00)	-	0.00 (0.00)
Jagatsukh	0.00	-	8.00 (16.37)	4.00 (8.18)
Thandidhar	23.33 (28.83)	26.67 (31.06)	8.33(16.72)	19.45 (25.54)
Rohru forest division	34.00(35.64)	-	13.33 (21.37)	23.67 (28.50)
Shillaru	35.00 (36.25)	48.00 (43.83)	10.00(18.27)	31.00 (32.79)
$Cd_{0.05}$				
1. Size categories with in populations		2.12		
2. Between population with number of size categories				
1 and 2		1.84		
1 and 3		1.73		
2 and 3		1.37		
2 and 2		1.50		
3 and 3		1.22		

Values in parentheses are Arc Sine transformed values.

Table 11. Impact of seed size on germination percentage.

Seed size	Germination%
Small	22.47 (25.48)
Medium	20.17 (23.16)
Large	12.73 (20.48)
CD0.05	NS*

* Non significant.

DISCUSSION

Morphology and floral architecture

The traded roots of *A. glauca* are sometimes adulterated by roots of *Pleurospermum angelicoides* (Wall. ex DC) Benth. ex C. B. Clarke and *Angelica archangelica* L., thereby making morphological studies crucial to check the genuiness of the species. Although the studied populations were of genuine *A. glauca* being similar in morphological features reported earlier (Clarke 1885; Kirtikar and Basu 1984; Bisht et al. 2003; Nautiyal and Nautiyal 2004; Vashistha et al. 2006), yet with regard to the sex type present observations have established beyond doubt presence of only bisexual flowers that has been reported earlier by Butola et al. (2010) also however, Bisht et al. (2008) have reported *A. glauca* as andromonoecious (both bisexual and staminate flowers on same individual). Apiaceae members exhibit diverse sexual expression with most of the species being andromonoecious, few bisexual (wild *Foeniculum vulgare* Mill.) and rest either dioecious (*Aciphylla* or *Anisotome*) or gynodioecious (*Gingidia*, *Scandia* and *Lignocarpa* etc.) (Koul et al. 1993; Reuther 2013).

Seed size variation corresponding to test weight is being reported for the first time in this species and such variation was observed irrespective of the umbel order. However, the primary umbel followed by lateral-I umbel only set seed with almost nil seed set by lateral-II and lateral-III umbels. This suggests that only two former types of umbels should be targeted for seed harvest. Seed size variation is also known in the Apiaceae species like *Anethum graveolens* L. and *Pastinaca sativa* L. wherein such variations is correlated with umbel order as well as the portion of flowers within an umbel (Hendrix 1984; Hołubowicz and Morozowska 2011).

Another important seed feature having implications for its reproductive fitness that has been observed is presence of seeds without embryo (thereby sterile) in the species. Low seed germination in *A. glauca* is already known and seeds without embryo probably are the reason. Low seed fertility due to the embryo less seeds may be the reason of its sporadic populations thereby leading to its rarity in nature. This is an important finding and any strategy towards sustainable utilization shall have to factor in this feature. This feature was irrespective of seed produced by different umbel order as well as pollination systems indicating physiological causes. Reduced fertile seed output may have some advantages like allowing enough space for progeny to grow but limit their number. In self incompatible *Stevia rebaudiana* Bertoni, two types of sterile and fertile seeds are produced, however that is due to genetic reasons (Raina et al. 2013).

Phenology

Phenology of *A. glauca* follows the general pattern of temperate perennial herbs that undergo perennation during winter period only to sprout back after snow melting. Flowering commences with the summer season with primary umbels blooming first followed by lateral-I, lateral-II and lateral-III umbels with peak flowering during August month. Seed maturation and shedding commences from last week of August month till October. Flowering is asynchronous among plants occurring in same niche and within plant too, different phenological events were asynchronous even among primary, lateral-I and lateral-II umbels which appears to be an adaptation to limited pollinator services especially insects. Vashistha et al. (2010) have also reported similar phenological events.

Floral biology

Flower of *A. glauca* have been observed to be protandrous with anther dehiscence beginning with the opening of floral buds that continues for 2-3 days. Stigma become receptive after complete anther dehiscence that is also characterized by style extending full beyond stylopodium indicating complete intra floral dichogamy. Shiny stylopodium is also indicator of stigma receptivity. Late maturation of stigma coupled with elongation of style after anther dehiscence facilitate dichogamy in *A. glauca* and appears as an adaptation to avoid autogamy as well as inbreeding depression. Protandry in *A. glauca* has also been reported by Bisht et al. (2008). In *Chaerophyllum bulbosum* L.(Apiaceae), also styles elongates only after pollen is shed and sexual phases are clearly distinguishable indicating extreme '*protandry*' (Reuther and Claßen-Bockhoff 2013). *Foeniculum vulgare* Mill. other member of Apiaceae, has also been found to be strongly '*protandrous*' as pollen are released much before stigma receptivity (Koul et al. 1996). As *A. glauca* thrives in hostile climatic condition, production of trinuclear pollen grains seems to be an adaptive feature for faster germination on stigma leading to reproductive assurance.

Pollen ovule ratio of 13064.37± 661.71, studied for the first time in present studies indicates the species to be an obligate outcrosser as per Cruden (1977). *Foeniculum vulgare*, another member of Apiaceae, is also characterized by high pollen ovule ratio of 12005-14635 (Koul et al. 1996).

Chromosomal studies

The present gametic chromosome count of n=11 is in conformity with the previous diploid count of 2n=22 (Kumar and Singhal 2011) from northwest Himalaya. However, grouping of bivalents at diakinensis and metaphase-I stage into a ring structure has been observed for the first time in this species. In, only few cells could clear 11 bivalents be observed at these stages. Anaphase-I too was characterized by the presence of two rings of 11 chromosomes at each poles. Presence of ring of 11 chromosomes at anaphase-I in *A. glauca* poles appears to be similar to the 'renner' complexes (entire haploid genomes which are inherited as single units) present in genus *Oenothera*, wherein due to reciprocal translocations of chromosome arms, all the 14 chromosomes form two rings of seven chromosome each (Greiner 2008).

As has been discussed earlier, significant proportion of seeds of *A. glauca* were without embryo leading to reduced germination, which may be due to this meiotic anomaly. Further studies on female meiosis, embryo development as well as more extensive studies on male meiosis are required to establish the consequences of meiotic anomaly in *A. glauca*. Although meiotic abnormalities like laggards, bridges, etc. were not observed but in some pollen mother cells, cytomixis was observed in *A. glauca*. Cytomixis often leads to abnormal meiotic behavior, variation in pollen grains size and low pollen viability or sterility e.g. in *Alopecurus arundinaceus* Poir. (Koul 1990), *Polygonum tomentosum* Willd. (Haroun 1995), *Hordeum vulgare* L.(Haroun 1996), *Brassica napus* var. *Oleifera* Delile. and *B. campestris* var. *oleifera* DC (Souza and Pagliarini 1997), *Vicia faba* L. (Haroun et al. 2004), and *Meconopsis aculeate* Royle (Singhal et al. 2008). Despite chromosome arranged in rings as well as cytomixis, pollen stainability did not seem to be impacted much as it ranged from 76% to 100% in different plants of *A. glauca* studied.

Breeding system studies

Significant seed set under open pollination conditions in comparison to autogamous conditions established the species strongly favouring (about 95%) cross pollination. This aspect is being reported for the first time in this species. Interestingly open pollination also resulted in much higher seed (with embryo) set (27.49 ± 2.67%) as compared to autogamous seed (1.27 ± 0.88%), again indicating strong presence of cross pollination.

As it is generally presumed that selfing rates increase with increasing altitudes (Schroter 1926; Bliss 1962; Garcia-Camacho and Totland 2009; Korner and Paulsen 2009) as pollinator abundance and activity become limiting factors due to hostile climatic conditions at higher altitudes (Arroyo et al.1982, 2006; Bingham and Orthner 1998; Medan et al. 2002). Contrary to this view, the species under investigation (*A. glauca*) favours cross pollination

and dichogamy seems to play a key role in its cross fertilization. *Eritricium nanum* (Vill.) Schrad.ex Gaudin (Boraginaceae), *Chaetanthera renifolia* (J. Remy) Cabrera (Asteraceae) and *Nardostachys grandiflora* DC (Valerianaceae) other temperate plant species, are also known for higher cross pollination rates at high altitudes (Writh 2010; Diaz et al. 2011; Gautam and Raina 2016). Inflorescence attributes, high pollen ovule ratio and asynchronous opening of flowers in *A. glauca* are also evidence for its cross pollinating nature. However, low seed set in *A. glauca* limits, natural variation essential for genetic improvement.

Seed biology

As has been already discussed only primary and lateral-I umbels set seeds. Among these too primary umbel set significantly more (56.71 ± 5.52%) seed than lateral-I umbel (20.08 ± 1.75%). Higher seed set in primary umbel in *Eryngium alpinum* L. and *Carum carvi* L. (family Apiaceae) is already known (Bouwmeester and Smid 1995; Gaudeul and Bottraud 2004).

Interestingly, despite blooming, lateral-II as well as lateral-III umbels do not set any seed which seems due to their late development or restricted resource allocation and they seem to only for enhancing floral visibility of its plants for pollinator attraction. Of the 56.71± 5.52% and 20.08 ± 1.75% seed set by primary and lateral-I umbels only27.49 ± 2.67% and 6.53 ± 0.57% seed is with embryo respectively indicating higher fertile seed production by primary umbels. This low fertile seed production in *A. glauca* may be the reason for generally low germination rates in this species.

Fruit and seed set percentage is generally low in late blooming flowers than early blooming ones (Zimmerman and Aide 1989), and several reasons like resource competition among the ovaries of an inflorescence (Lee 1988); reduced pollen receipt by later blooming inflorescence (Lee 1988); intrinsic features (Berry and Calvo 1991; Diggle 1995) may be the reasons.

Non significant variations in fertile seed production between peripheral and central flowers of a umbel and also among seed from two ecologically different populations (Shilly and Shillaru) indicates that *A. glauca* as a strategy, limits fertile seed production either for nutrient resource conservation or to ensure better quality fertile seed that can produce a healthy progeny.

Seed germination

Seed germination behavior of any species impacts the genetic variability in any species and lower germination rates deprive such variation. Inter population germination variation was observed amongst the six population viz. Khan Jungle, Kilba, Jagatsukh, Thandidhar, Rohru forest division and Shillaru with Shillaru population excelling others (31.00% seed germination) with no germination in seeds of Kilba.

Apiaceae family members are known for generally low germination rates (Koul et al. 1993) and in *A. glauca* poor and erratic germination with maximum of 8% germination is already reported (Nautiyal et al. 2002; Butola and Budola, 2004; Butola and Samant 2006). Present studies have revealed that low germination in *A. glauca* is not due to dormancy but production of seeds without embryo.

CONCLUSION

Only primary and lateral-I umbels set seeds as other lateral-II and lateral-III only attract pollinators without setting any seed. Production of embryo less seeds (sterile seed) is a major reproductive bottleneck in this species. Seed size variation occurs within same plant as well as within same umbel with large seeds having higher proportion of fertile seeds (with embryo). The species is strongly cross pollinating.

ACKNOWLEDGEMENT

This study was funded by Department of Biotechnology (DBT), Government of India. The first author would like to acknowledge Department of Science and Technology (DST), Government of India for INSPIRE (Innovation in Science Pursuit for Inspired Research) Fellowship.Authors would also like to acknowledge Director, HFRI (Himalayan Forest Research Institute, Conifer Campus, Panthaghati, Shimla–171009, Himachal Pradesh, India) and Dr. Sandeep Sharma (Scientist–E, HFRI) for providing necessary facilities for conducting research experiments.

REFERENCES

Arroyo MTK, Munoz MS, Henriquez C, Bottraud TI, Perez F. 2006.Erratic pollination, high selfing levels and their correlates and consequences in an altitudinally widespread above tree-line species in the high Andes of Chile. Acta Oeco. 30(2): 248–257.

Arroyo MTK, Primack R, Armesto J. 1982.Community-studies in pollination ecology in the high temperate Andes of central Chile: pollination mechanisms and altitudinal variation. Am J Bot. 69(1): 82–97.

Berry PE, Calvo RN. 1991. Pollinator limitation and position dependent fruit set in the high Andean orchid *Myrosmodes cochleare* (Orchidaceae). Plant Syst Evol. 174(1): 93–101.

Bingham RA, Orthner AR. 1998.Efficient pollination of alpine plants. Nature. 391(6664): 238–239.

Bisht AK, Bhatt A, Rawal RS, Dhar U. 2008.Assessment of reproductive potential of different populations of *Angelica glauca* Edgew: a critically endangered Himalayan medicinal herb. J Mt Sci. 5(1): 84–90.

Bisht AK, Manjkhola S, Joshi M. 2003. Comparative account of two high value species of Himalayas: *Angelica glauca* Edgew. and *Angelica archangelica* L. Indian For.129 (10): 1241–1248.

Bliss LC.1962.Adaptations of arctic and alpine plants to environmental conditions. Arctic. 15(2): 117–144.

Bouwmeester HJ, Smid HG. 1995. Seed yield in caraway (*Carum carvi*): role of pollination. J of Agric Sci. 124(2): 235–244.

Butola JS and Badola HK. 2006. Chemical treatments to improve seedling emergence, vigour and survival in *Heracleum candicans* Wall. (Apiaceae): a high value threatened medicinal and edible herb of Himalaya. J. Plant Biol. 33(3): 215–220.

Butola JS, Badola HK. 2004. Effect of pre-sowing treatment on seed germination and seedling vigor in *Angelica glauca*: a threatened medicinal herb. Curr Sci. 87(6): 796–799.

Butola JS, Badola HK. 2008. Propagation conditions for mass multiplication of three threatened Himalayan high value medicinal herbs. P G R Newsletter. 153: 143–147.

Butola JS, Samant SS. 2006. Physiological studies on seed germination of *Angelica glauca*. J Trop Med Plants. 7(2): 205–212.

Butola JS, Vashistha RK, Samant SS, Malik AR. 2010. Technology for propagation and cultivation of *Angelica glauca* Edgew.: a threatened high value Himalayan medicinal cum edible herb. Emer Med Plants. 2(1): 67–72.

Butola JS, Vashistha RK. 2013. An overview on conservation and utilization of *Angelica glauca* Edgew. in three Himalayan states of India. Med Plants. 5(3):171–178

Chauhan NS. 1999. Medicinal and aromatic plants of Himachal Pradesh. New Delhi (India): Indus Publishing Company.

Clarke CB. 1885. The flora of British India. VolI. L. London: Reene and Co.

Corner EJH. 1976. Seeds of dicotyledons. Cambridge: Cambridge University Press.

Cruden RW. 1977. Pollen ovule ratio: a conservative indicator of breeding system in flowering plants. Evolution. 31(1):32–46.

Diaz CT, Gonzalez SG, Stotz GC, Morales PT, Paredes B, Millaqueo MP, Gianoli E.2011. Extremely long-lived stigmas allow extended cross-pollination opportunities in high Andean plant. PLoSONE. 6(5): e19497. doi:10.1371/journal.pone.0019497.

Diggle PK. 1995. Labile sex expression of andromonoecious *Solanum hirtum*: pattern of variation in floral structure. Can J Bot. 69(9): 2033–2043.

Garcia-Camacho R, Totland O. 2009. Pollen limitation in the alpine: a meta-analysis. Arct Antarct Alp Res. 41(1):103–111.

Gaudeul M, Bottraud IT. 2004. Reproductive ecology of the endangered alpine species *Eryngium alpinum* L. (Apiaceae): phenology, gene dispersal and reproductive success. Ann Bot. 93(6):711–721.

Gautam K, Raina R. 2016.New insights into the phenology, genetics and breeding system of critically endangered *Nardostachys grandiflora* DC. Caryologia. 69 (1): 91–101.

Gomez KA, Gomez AA. 1984. Statistical procedures for agricultural research. 2nd edition. New York (John Wiley).

Goswami PK, Khale A, Ogale S. 2012.Natural remedies for polycystic ovarian syndrome (PCOS): a review. Inter J Pharmaceut and Phytopharmaco Res. 1(6): 396–402.

Greiner S. 2008. Oenothera, a unique model to study the role of plastids in speciation, Dissertation der Fakultätfür Biologie der Ludwig-Maximilians-Universität München (Dissertation at the Faculty of Biology, at the Ludwig Maximilian University of Munich).

Haroun SA, Al Shehri AM, Al Wadie HM. 2004. Cytomixis in the microsporogenesis of *Vicia faba* L. (Fabaceae).Cytologia. 69(1): 7–11.

Haroun SA. 1995. Cytomixis in the pollen mother cells of *Polygonatum tomentosum* Schrank. Cytologia. 60(3): 257–260.

Haroun SA. 1996. Induced cytomixis and male sterility in pollen mother cells of *Hordeum vulgare* L. Delta J Sci. 20(1): 172–183.

Hendrix SD. 1984. Variation in seed weight and its effects on germination in *Pastinaca sativa* L. (umbelliferae). Am J Bot. 71(6): 795–802

Hołubowicz R, Morozowska M. 2011.Effect of umbel position on dill (*Anethum graveolens* L.) plants growing in field stands on selected seed stalk features. Folia Hort. 23(2): 157–163.

IUCN.1993. Draft IUCN red list categories. Gland, Switzerland.

Kirtikar KR, Basu BD. 1984.Indian Medicinal Plants.2nd edition. Dehradun (India).Bishen Singh Mahendra Pal Singh, 1592 p.

Korner C, Paulsen J. 2009. Exploring and explaining mountain biodiversity. In: Spehn M and Korner C, Editors. Data mining for global trends in mountain biodiversity. Florida (USA): CRC Press, 10 p.

Koul K K. 1990. Cytomixis in pollen mother cells of *Alopecurus arundinaceus* Poir. Cytologia. 55(1): 169–173.

Koul P, Sharma N, Koul AK. 1993. Pollination biology of Apiaceae. Curr. Sci. 65(3): 219–222.

Koul P, Sharma N, Koul AK. 1996. Reproductive biology of wild and cultivated fennel (*Foeniculum vulgare* Mill.). Proc Indian Natn Sci Acad. B62 (2): 125–134.

Kuafman PB, Carison TF, Dayanadan P, Evans ML, Fisher JB, Parks O, Wells JR. 1989.Plants, their biology and importance. New York (USA): Hopper and Raw Publishers.

Kumar P, Singhal VK. 2011. Chromosome number, male meiosis and pollen fertility in selected angiosperms of the cold deserts of Lahaul-Spiti and adjoining areas (Himachal Pradesh, India).Plant Syst and Evol. 297(3-4): 271–297.

Lawrence GHM. 1951.Taxonomy of vascular plants. New York: McMillan.

Lee TD. 1988. Patterns of fruit and seed production. In: Doust J L and Doust L L, Editors, Plant reproductive ecology: patterns and strategies. New York (USA): Oxford University Press, 179–202 p.

Medan D, Montaldo NH, Devoto M, Mantese A, VasellatiV, Bartoloni NH. 2002. Plant–pollinator relationships at two altitudes in the Andes of Mendoza, Argentina. Arct Antarct Alp Res. 34(3): 233–241.

Nautiyal MC, Prakash V, Nautiyal BP .2002. Cultivation techniques of some high altitude Medicinal herbs. Ann For. 10(1): 62–67.

Nautiyal MC, Nautiyal BP. 2004.Agrotechniques for high altitude medicinal and aromatic plants. High Altitude Plant Physiology Research Centre, Dehradun, India, pp. 99–133.

Pimenov MG, Leonov MV. 2004. The Asian Umbelliferae biodiversity database (ASIUM) with particular reference to South-West Asian taxa. Turk J Bot. 28(1–2): 139–145.

Pimenov, MG, Kljuykov EV. 2003. Notes on some Sino-Himalayan species of *Angelica* and *Ostericum* (Umbelliferae). Willdenowia 33: 121–137.

Raina R, Bhandari SK, Chand R, Sharma YP. 2013. Strategies to improve poor seed germination in *Stevia rebaudiana*: a low calorie sweetener. J of Med Plants Res.**7**(24): 1793–1799.

Reuther K, Claßen-Bockhoff R. 2010.Diversity behind uniformity: inflorescence architecture and flowering sequence in Apiaceae-Apioideae. Plant Div and Evol. 128(1–2): 181–220.

Reuther KU. 2013. Spatial and temporal flower presentation in Apiaceae-apioideae. Dissertation, Doctor of Science, Department of Biology at the University of Mainz.

Saeed MA, Sabir AW. 2008. Irritant and cytotoxic coumarins from *Angelica glauca* Edgew Roots. J Asian Nat Prod Res. 10(1): 49–58.

Samant SS, Dhar U, Palni LMS. 1998. Medicinal plants of Himalaya: diversity distribution and potential values. Nainital, India, Himvikas, GyanodayaPrakashan.

Samant SS, Nandi SK, Butola JS.2009.Conservation status and cultivation of selected medicinal plants in the Indian Himalayan Region. In: Chaurasia SB, Yadav OP, Rimando, AM, Terrill, TH. Editors. Advances in Agriculture, Environment and Health: Fruits, Vegetables, Animals and Biomedical Sciences. Delhi (India): Satish Serial Publishing House. pp 185–214.

Schroter C. 1926. *Das Pflanzenleben der Alpen* [The plant life in the Alps]. Albert Raustein, Zurich, Switzerland.

Sher H, Elyemeni M, Kamran H and Sher H. 2011. Ethnobotanical and economic observations of some plant resources from the northern parts of Pakistan, Ethnobot Res Appl. 9: 027–041.

Singh SK, Rawat GS. 2000. Flora of great Himalayan national park, Himachal Pradesh, Dehradun (India) Bishen Pal Singh and Mahendra Pal Singh.

Singhal VK, Kumar P. 2008.Impact of cytomixis on meiosis, pollen viability and pollen size in wild populations of Himalayan poppy (*Meconopsis aculeata* Royle).J Biosci. 33(3): 371–380.

Souza AM, Pagliarini MS. 1997.Cytomixis in *Brassica napus* var. *oleifera* and *Brassica campestris* var. *Oleifera* (Brassicaceae).Cytologia. 62(1): 25–29

The wealth of India: a dictionary of Indian raw materials and industrials products. 1985. Council for Scientific and Industrial Research, I. A. Public and Information Directorate, New Delhi, India, 153 p.

Vashistha R, Nautiyal BP, Nautiyal MC. 2006.Conservation status and morphological variations between populations of *Angelica glauca* Edgew. and *Angelica archangelica* Linn. in Garhwal Himalaya. Curr Sci. 91(11): 1537–1542.

Vashistha RK, Butola JS, Nautiyal BP, Nautiyal MC. 2010. Phenological attributes of *Angelica glauca* and *A. archangelica* expressed at two different climatic zones in Western Himalaya. Open Access J Med Arom Plants. 1(1): 7–12.

Weberling F.1989. Morphology of flower and inflorescence. New York (USA) Cambridge University Press.

Wirth LR, Graf R, Gugerli F, Landergott U, Holderegger R. 2010. Lower selfing rate at higher altitudes in the alpine plant *Eritrichium nanum* (Boraginaceae). Am J Bot. 97: 899–901.

Zimmerman J K and Aide T M.1989. Patterns of fruit production in a neotropical orchid: pollinator vs. resource limitation. Am. J Bot. 76(1):67–73.

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

Citation: A. Ždralović, A. Mesic, I. Eminović, A. Parić (2019) Cytotoxic and genotoxic activity of *Plantago major* L. extracts. *Caryologia* 72(3): 35-40. doi: 10.13128/caryologia-759

Published: December 13, 2019

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

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

Cytotoxic and genotoxic activity of *Plantago major* L. extracts

Amira Ždralović, Aner Mesic, Izet Eminović, Adisa Parić*

Department of Biology, Faculty of Science, University of Sarajevo, Zmaja od Bosne 33-35, 71000 Sarajevo, Bosnia and Herzegovina
*Correspondence to: e-mail: adisa.p@pmf.unsa.ba

Abstract. *Plantago major* L. is a perennial, wild plant that belongs to the Plantaginaceae family, and is used as a good indicator in the assessment of destructive anthropogenic impact on the environment. The aim of the present study was to evaluate cyto/genotoxic effects of methanol extracts of *Plantago major*, collected from two locations (Tetovo and Smetovi), using *Allium cepa* test. We demonstrated that the highest concentration of *P. major* extracts from both locations reduced the mitotic index, while the lowest increased mitotic index value comparing to the positive control. As for genotoxic effects of extract from Tetovo, all concentrations increased the frequency of sticky chromosomes and chromosome missegregations in comparison with both controls, and frequency of multipolar anaphases when compared to the negative control. Higher number of cells with vagrants in comparison with positive control was detected after the treatment with 0.005 and 0.02 mg/ml concentrations. *P. major* extract from Smetovi (0.005 and 0.01 mg/ml) induced an increase in the number of vagrants as compared to the positive control, and frequency of sticky chromosomes when compared to both controls (0.01 mg/ml). Exposure to extract (0.005 and 0.02 mg/ml) caused increased number of multipolar anaphases in comparison with negative control. Apoptosis were not detected for *P. major* extract from Smetovi, while its highest concentration (0.02 mg/ml) induced increase in the frequency of necrosis as compared to the positive control. Our results demonstrated that methanol extracts of *P. major*, collected from Tetovo and Smetovi, showed cyto/genotoxic effects on *A. cepa* meristem cells.

Keywords. *Plantago major*, Cyto/genotoxicity, *Allium cepa* test, Heavy metals.

INTRODUCTION

Plantago major L. is herbaceous, perennial wild plant from the family Plantaginaceae that is distributed throughout the world (Samuelsen 2000; Thome et al. 2012). Low growth and growth in the form of rosettes make this type well-adjustable on trampling, grazing and mowing (Thomet 1978). *P. major* contains biologically active compounds such as polysaccharides, lipids, phenols, flavonoids, iridoid glycosides, terpenoids (Samuelsen 2000; Chiang et al. 2003), benzoic compounds (Chiang et al. 2003), tannins, saponins and sterols (Jurišić Grubešić et al. 2005). Recent research shows that plants used in traditional medicine consumption exhibit mutagenic, geno-

toxic and cytotoxic effects *in vitro* and *in vivo* (Higashimoto et al. 1993; Schimmer et al. 1994; Kassie et al. 1996; Aşkin Çelik and Aslantürk 2007). Many chemical plant constituents have the ability to react with the DNA molecule, and may cause damage in DNA structure and/or disruption of biochemical reactions (Sofradžija et al. 1989). In order to reduce the risk of application of natural agents, plants and their parts, as well as plant extracts, it is necessary to assess their ability to induce cytotoxic, genotoxic and mutagenic effects (Askin Celik 2012). An indispensable aspect that must be noted in the modern use of plants for medicinal purposes is the increasing pollution of the environment by human activity. Plants actively participate in the circulation of nutrients and gases such as carbon dioxide, oxygen and also provide a big surface area for absorption and accumulation of air pollutants reducing the level of pollutants in the environment (Escobedo et al. 2008). Numerous studies have shown that *P. major* L. is good indicator of the degree of destructive anthropogenic impact on the environment (Montacchini and Siniscalco 1979). Furthermore, it has been demonstrated that methanol extracts of *P. major* demonstrated cytotoxic activity in different cancer cell lines (Kartini et al. 2017). *Allium cepa* assay has been described as an efficient test used for genotoxicity assessment of potential genotoxic agents in the samples taken from the environment, due to its sensitivity and good correlation with mammalian test systems *in vitro* (Firbas and Amon 2014; Prajitha and Thoppil 2016). Therefore, the aim of this research was to examine the cyto/genotoxic effects of methanol extracts of *P. major* sampled from Tetovo (polluted location) and Smetovi (control location) using *Allium cepa* test.

MATERIALS AND METHODS

Plant material

Plantago major was collected on two locations: Tetovo, which is near ironworks ArcelorMittal (exposed to daily air pollution, dust, sulfur dioxide and other pollutants) and Smetovi, located at 1.025 meters above sea level and is a popular resort and hiking destination. Plant samples were collected in October 2015. For testing were used leaves of the plant, which were carried out on the same day at both sites in PVC packaging bags and within 3 hours of collection delivered in the Laboratory of Plant Physiology, Department of Biology on Faculty of Science, University of Sarajevo. Plant material was dried at room temperature, away from direct light and stored at + 4°C until analysis. Voucher specimens (No. LRPER 383-384) were deposited in the Laboratory for research and protection of the endemic gene pool.

Extraction procedure

To prepare the plant extracts we used dry plant material and 80% methanol as solvent. 1 g of plant material was chopped and mixed with 40 ml of methanol. Incubation period was 24 h at 4°C. After filtration, supernatant was evaporated to a dry residue which was re-dissolved in 80% methanol in three concentrations: 0.02; 0.01; 0.005 mg/ml. Along three concentrations of extracts (from each location), we tested two controls: positive (80% methanol) and negative (distilled H_2O).

Allium test

For detection of cyto/genotoxic effects of *P. major* L. extracts, *Allium cepa* bulbs were used. Onion bulbs were grown in the glass vessels filled with tap water and left for germination for 48 h at room temperature with water injection as needed. We selected four bulbs for each treatment and measured their root length as previously described by Fiskesjo (1993). In this sense, the length of the root bundles from each onion bulbs was measured. The measure is taken from the point where the roots sprout, down to where the most root tips end their growth. Afterward, the bulbs were treated with *P. major* L. extracts for 24h at room temperature. At the end of the exposure period the root lengths of the bulbs were measured. For each treatment the bulb root is removed and placed into the appropriate labeled tubes containing ethanol/glacial acetic acid (3:1, v/v) fixative and kept for 24h at 4°C.

Cytogenetic analysis

A. cepa roots were hydrolyzed in 1M HCl solution for 15 minutes at room temperature. After that, the roots were transferred to distilled water. The apical 2 mm of the root were cut and placed in one drop of 2% acetorcein and squashed. Microscope slides were analyzed under the light microscope with a magnification of 400x. All photographs were made by use of Sony Cyber shoot ISO 3200 camera. To calculate the mitotic index values, 1000 cells per slide was analyzed. Chromosomal aberrations were analyzed on 100 cells in division per treatment. Counting 1000 interphase cells for each concentration, the frequency of micronuclei was determined. Apoptosis and necrosis were analyzed at 1000 interphase cells per slide.

Table 1 Roots length of *Allium cepa* (mean ± SD) before and after the treatment with different concentrations of the tested *P. major* extracts.

Concentration (mg/ml)	Tetovo		Smetovi		Methanol		H_2O	
	Roots length[a]	Roots length[b]	Roots length[a]	Roots length[b]	Roots length[c]	Roots length[d]	Roots length[c]	Roots length[d]
0,005	1.90±0.52	1.87±0.47	1.52±0.15	1.62±0.12*				
0,01	2,02±0.17	2,02±0.17	1.87±0.68	1.92±0.65	1,56±0,56	1,56±0,56	1.80±0.29	2,50±0.33
0,02	1.72±0.25	1.67±0.18*	1.70±0.73	1.70±0.74				

*Statistically significant difference compared to the negative control ($P < 0.01$).
Values are expressed in centimeters.
SD: Standard deviation.
[a] Root length in the first 48 h before treatment with different concentrations of the extracts.
[b] Root length in the next 24 h after treatment with different concentrations of the extracts.
[c] Root length of the control group in the first 48 h.
[d] Root length of the control group in the following 24 h.

Statistical analysis

To evaluate differences between tested concentrations and controls, for all analyzed parameters, Student's t-test was used. All statistical analyses were conducted by use of Microsoft Excel 2007 (Microsoft Corporation) and SPSS 20.0 software (SPSS, Chicago, IL). P values less than 0.05 were considered statistically significant.

RESULTS

The most important macroscopic parameter in *Allium* test is a root length (Fiskesjö 1985). Different concentrations of *P. major* extracts had different effects on root growth (Table 1). The extracts of *P. major* from polluted location inhibited root growth. Statistically significant effect had the highest concentration (0.02 mg/ml) of extract from mentioned location when compared to the control plants. Concentration of the extract of *P. major* (0.005 mg/ml) significantly stimulated root growth as compared to the negative control.

Table 2 The mitotic index of *Allium cepa* meristematic cells (mean ± SD) exposed to various concentrations of the samples

Concentration (mg/ml)	Tetovo	Smetovi	Methanol	H_2O
0,005	2,10±0.69	4.57±0.49**		
0,01	1.27±0.63	2.92±0.94	2.05±0.51	2.65±2.14
0,02	1.65±1.02	0.95±0.65*		

**Statistically significant difference compared to the positive control (methanol) ($P < 0.001$).
Values are expressed as a percentage.
SD: Standard deviation. MI: Mitotic index.
*Statistically significant difference compared to the positive control (methanol) ($P < 0.05$).

In order to evaluate the effect of *P. major* extracts, mitotic activity of *A. cepa* meristem cells was expressed as a percentage of cells in division in relation to the total number of analyzed cells. Statistically significant difference compared to control, was observed in the treatment with *P. major* extract from Smetovi (0.02 mg/ml, and 0,005 mg/ml) (Table 2). The highest concentrations of *P. major* extracts (0.02; 0.01 mg/ml) from Tetovo lead to the reduction in mitotic activity compared to the control, but the difference was not statistically significant.

The results of the genotoxic and cytotoxic effects of different concentrations of the *Plantago major* extract from two locations are presented in Table 3. Several genotoxic effects were observed, such as micronuclei, sticky chromosomes, anaphase bridges, vagrant chromosomes, chromosome missegregation, multipolar anaphases, as well as apoptotic and necrotic cells as cytotoxicity endpoints.

P. major extract from Tetovo (0.005 mg/ml) caused the highest number of chromosome aberrations. Similar effects were observed for extract from same location in concentration of 0.02 mg/ml. In this sense, micronuclei (Fig. 1a), multipolar anaphase (Fig. 1b), sticky chromosomes (Fig. 1c), anaphase bridges (Fig. 1d), vagrant chromosomes (Fig. 1e), chromosome missegregation, were observed. The highest number of apoptotic and necrotic cells (Fig. 1f) is observed after the treatment with the highest concentration (0.02 mg/ml) from this location, while apoptotic cells were not detected at the lowest concentration.

Unlike Tetovo, the frequency of chromosomal aberrations on meristem cells that were treated with extract of *P. major* from Smetovi (non-polluted place), was much lower. Chromosome aberrations that have reached statistical significance are: sticky chromosomes, vagrant chromosomes and multipolar anaphases. On

Table 3 The results of the genotoxicity and cytotoxicity in *Allium cepa* (mean ± SD) exposed to various concentrations of the *P. major* extract.

Tested extracts (mg/ml)	Micronuclei	Sticky chromosomes	Abnormal ana/telophases				Apoptotic cells	Necrotic cells
			Bridges	Vagrant	Chromosome missegregation	Multipolarity		
Tetovo								
0,005	0.75±0.95	5±3.16 ^{**2}	2±1.41^{*1}	2.5±1.29**	4±1.41^{**2}	9.25±2.87^{3}	n.o.	2±1.82
0,01	n.o.	4.50±3^{*1}	0.25±0.50	2.75±2.98	3±1.15^{**2}	7.75±3.50^{2}	0.50±1	1.75±1.25
0,02	0.50±0.57	2±1.15^{**2}	1.25±1.25	1.50±1.29*	2.50±1^{**2}	7.50±2.51^{3}	0.50±0.57	7±2.16
Smetovi								
0,005	n.o.	0.50±0.57	0.75±0.95	2.50±1.29*	0.25±0.50	5.25±1.50^{3}	n.o.	1.75±2.36
0,01	n.o.	2.75±2.21^{*1}	1±1.41	2.50±1.91*	1.50±0.57	5.50±0.57	n.o	1±2
0,02	0.25±0.50	0.75±0.95	0.50±0.57	3±3.82	1±0.57	7.75±6.02^{1}	n.o.	0.25±0.50*
Methanol	n.o.	n.o.	n.o.	n.o.	0.25±0.5	4.25±3.30	n.o.	4.50±3.10
H_2O	n.o.	n.o.	n.o.	1±0.81	0.25±0.5	n.o.	n.o.	5.25±4.57

Statistically significant difference compared to the negative control (H_20): ^{1} P < 0.05, ^{2} P < 0.01; ^{3} P < 0.001.
SD: Standard deviation.
Statistically significant difference compared to the positive control (methanol) after the T-test: * P < 0.05; ** P < 0.01.

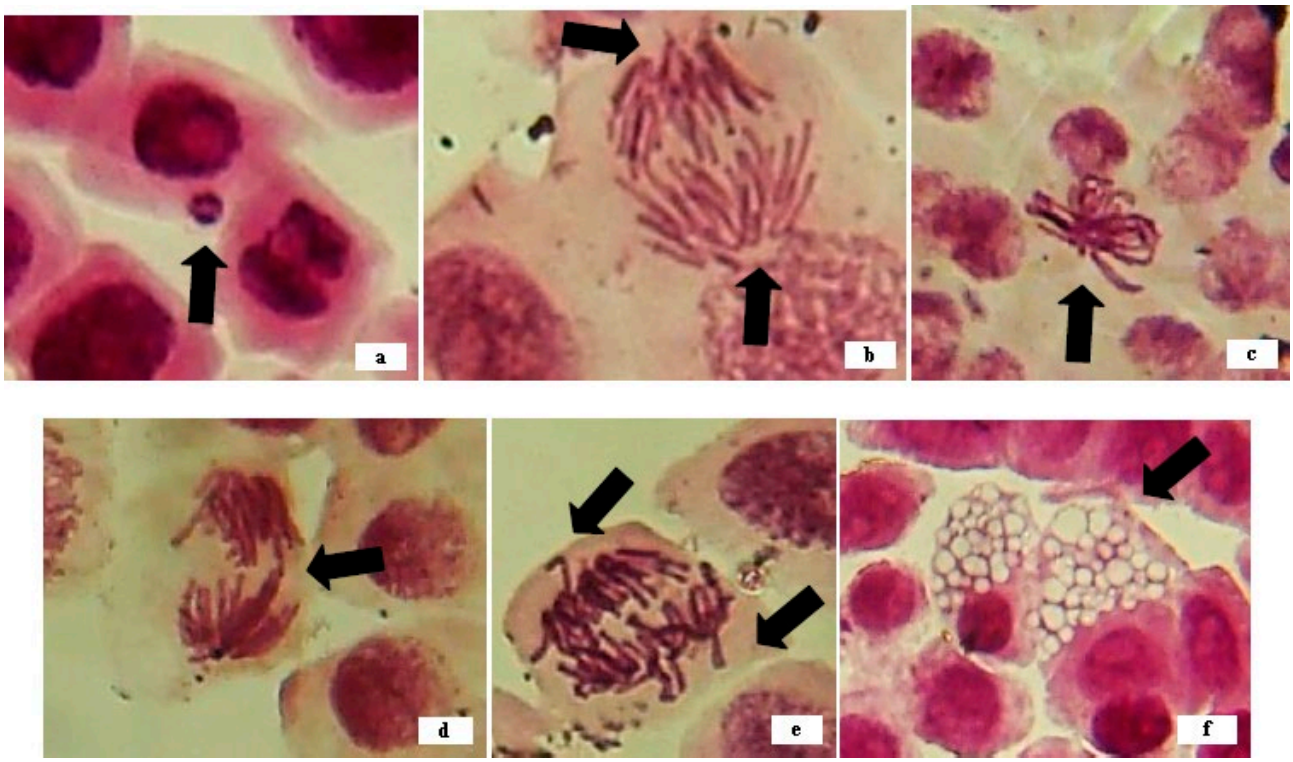


Fig. 1 Genotoxic effects of *Allium cepa* meristem cells treated with *P. major* extracts from Tetovo: a- micronucleus, b- multipolar anaphase, c- sticky chromosomes, d- anaphase bridge, e- vagrant chromosomes, f- necrotic cells.

the meristem cells treated with *P. major* extract from this location, apoptotic changes were not observed, while necrotic cells were few, but the numbers were statistically significant only for 0,02 mg/ml concentration (because of low number of necrotic cells compared to the positive control).

DISCUSSION

Plant species are an excellent source of biologically active substances, whose effects on genetic material are largely unknown (Barnes 2003). Toxicity is easy to see in inhibition of root growth, while mutagenicity correlates with chromosomal aberrations (Fiskesjö 1985). Inhibition of root growth is always parallel with the decline in cell division (Fiskesjö 1997), and can be caused by heavy metals in the plant extract. It has been found that the toxicity of extracts from plants which contain heavy metals, such as manganese, cadmium and lead is often associated with these pollutants (Boroffice 1990; Fiskesjö 1997). Recent study from locations Tetovo and Smetovi demonstrated that *P. major* is exposed to the negative impact of heavy metals, particularly in the area of Tetovo (Muratovic 2016). Heavy metals imply inhibition of *A. cepa* root growth. Early researches of plant tolerance to the heavy metals have shown that root growth is particularly sensitive to the presence of metal toxins. As a result of root growth cytokinesis, cell differentiation and extensions, metal induced inhibition of root growth is a result of toxic influences, acting on any of the three processes (Baker and Walker 1989). Rajeshwari et al. (2015) proved that aluminum nanoparticles increased the number of chromosomal aberrations in the *A. cepa* root tip cells and similar results were observed for other heavy metals such as Cu, Pb, Fe, Cd, Ni, Zn etc. (Olorunfemia et al. 2015). Therefore, our results suggest that inhibited growth of roots that were treated with *P. major* extract from polluted area of Tetovo could be due to presence of the heavy metals in plants.

The cytotoxicity of some chemical component, or plant extracts can be determined based on the increase or decrease in the mitotic index (Smaka-Kincl et al. 1996). Reduction of mitotic activity can arise as a result of inhibiting the synthesis of DNA molecules in cells or by stopping the G2 phase of the cell cycle through the action of various toxic substances present in plant extracts (Sudhakar et al. 2001). In this regard, it is important to accentuate that reduction in mitotic activity is parallel with the root growth inhibition of *A. cepa* meristem cells, which were observed after exposure to *P. major* extracts (0.02; 0.01 mg/ml) from Tetovo.

Similar results were observed by Askin Çelik & Aslantürk (2006) with reduction of mitotic index induced by *Plantago lanceolata* L. extracts, which indicates that the substance in the aqueous extract can have a cytotoxic effect. It is proven that extract of *P. major* reduce cell proliferation *in vitro* (Samuelsson 2004). Extracts of *Plantago* species have a cytotoxic effect on different tumor cell lines (Richardson 2001) due to the presence of luteolin 7-O-β-glucoside, as the main flavonoid present in most *Plantago* species (Galvez et al. 2003).

Comparing the genotoxic effects of these two locations, we can see that the total number of chromosomal aberrations was higher in cells treated with extract of *P. major* from Tetovo, which was expected because of air pollutants source. Smetovi is well known as an excursion site, and perceived aberrations (vagrant and sticky chromosomes) on this location could be explained by gasses from motor vehicles and the presence of waste material.

The toxicity of metals in the plant can be manifested with few biological markers that can be detected and analyzed at different levels of the organization, morphology of the plant as well as at the biochemical and molecular level. Therefore, they are very useful for plant monitoring and assessment of the environmental pollution (Olorunfemia et al. 2015). Among various biological effects which could be consequence of environmental pollution, genotoxicology is one aspect that is related to DNA damage and genome. According to this, our results are of great value in terms of use of *Plantago major* as an indicator of environmental pollution with heavy metals and other toxic substances.

In conclusion, the results of the present study revealed that *P. major* extracts from polluted location Tetovo reduced root growth and mitotic activity of *A. cepa* meristem cells, and that possess significant cyto/genotoxic potential. Observed chromosomal aberrations indicate that *P. major* extracts exhibit clastogenic properties with ability for mitotic spindle disruption, implying that *P. major* could be very useful in monitoring of the environmental pollution. Further toxicological studies on animal models are needed to strengthen these findings.

REFERENCES

Aşkin Çelik T, Aslantürk Ö. 2006. Anti-mitotic and antigenotoxic effects of *Plantago lanceolata* aqueous extract on *Allium cepa* root tip meristem cells. Biologia. 61(6): 693-697.

Aşkin Çelik T, Aslantürk ÖS. 2007. Cytotoxic and genotoxic effects of *Lavandula stoechas* aqueous extracts, Biologia. 62(3): 292-296.

Askin Celik T. 2012. Potential genotoxic and cytotoxic effects of plant extracts. In: Bhattacharya A, editor. A compendium of essays on alternative therapy. London: InTech; p. 302.

Baker AJM, Walker P. 1989. Physiological responses of plants to heavy metals and the quantification of tolerance and toxicity. Chem. Speciation Bioavailability. 1(1): 7-17.

Barnes J. 2003. Pharmacovigilance of herbal medicines: a UK perspective. Drug Saf. 26 (12): 829-851.

Boroffice RA. 1990. Cytogenetic effects of zinc and chromium on the onion (*Allium cepa*) root tip. Niger J. Nat. Sci. 1: 75-79.

Chiang L, Chiang W, Chang M, Lin C. 2003. In vitro cytotoxic, antiviral and immunomodulatory effects of *Plantago major* and *Plantago asiatica*. Am J Chin Med. 31(2): 225-234.

Escobedo F J, Wagner J E, Nowak DJ, De la Maza CL, Rodriguez M, Crane DE. 2008. Analyzing the cost effectiveness of Santiago, Chile's policy of using urban forests to improve air quality. J Environ Manage. 86(1): 148-157.

Firbas P, Amon T. 2014. Chromosome damage studies in the onion plant Allium cepa L. Caryologia. 67(1): 25-35.

Fiskesjö G. 1985. The Allium test as a standard in environmental monitoring. Heraditas. 102: 99-112.

Fiskesjö G. 1993. *Allium* test I: A 2-3 day plant test for toxicity assessment by measuring the mean root growth of onions (*allium cepa* L.). Environ Toxicol. 8(4): 461-470.

Fiskesjö G. 1997. Allium test for screening chemicals; evaluation of cytologic parameters. In: Wang W, Gorsuch JW, Hughes JS. Editors. Plants for Environmental Studies. Boca Raton, New York: CRC Lewis Publishers.

Galvez M, Martı́ C, Lopez-Lazaro M, Cortes F, Ayus J. 2003. Cytotoxic effect of Plantago spp. on cancer cell lines. J Ethnopharmacol. 88(2): 125-130.

Higashimoto M, Purintrapiban J, Kataoka K. 1993. Mutagenicity and antimutagenicity of extracts of three spices and a medicinal plant in Thailand. Mutat Res. 303(3): 135-142.

Jurišić Grubešić R, Vuković J, Kremer D, Vladimir-Knežević S. 2005. Spectrophotometric method for polyphenols analysis: Prevalidation and application on *Plantago* L. species. J Pharm Biomed Anal. 39(3–4): 837-842.

Kartini, Piyaviriyakul S, Thongpraditchote S, Siripong P, Vallisuta O. 2017. Effects of *Plantago major* extracts and its chemical compounds on proliferation of cancer cells and cytokines production of lipopolysaccharide-activated THP-1 Macrophages. Pharmacogn Mag. 13(51): 393-399.

Kassie F, Parzefall W, Musk S, Johnson I, Lamprecht G, Sontag G, Knasmüller S. 1996. Genotoxic effects of crude juices from Brassica vegetables and juices and extracts from phytopharmaceutical preparations and spices of cruciferous plants origin in bacterial and mammalian cells. Chem Biol Interact. 102(1): 1-16.

Montacchini F, Siniscalco C. 1979. The effects of foot traffic on vegetation and soils of city park turfs. Annali della Facolta di Scienze Agrarie della Universita degli Studi di Torino. 12: 365-385.

Muratovic D. 2016. *Sadržaj teških metala u listovima Plantago major L. sa područja Zenice*, Završni rad I ciklusa, Univerzitet u Sarajevu: Prirodno-matematički fakultet.

Olorunfemia DI, Duru L, Olorunfemib OP. 2015. Genotoxic effects of bilge water on mitotic activity in *Allium cepa* L. Caryologia. 68(4): 265–271.

Prajitha V, Thoppil JE. 2016. Genotoxic and antigenotoxic potential of the aqueous leaf extracts of *Amaranthus spinosus* Linn. using *Allium cepa* assay. S Afr J Bot. 102: 18-25.

Rajeshwari A, Kavitha S, Alex SA, Kumar D, Mukherjee A, Chandrasekaran N, Mukherjee A. 2015. Cytotoxicity of Aluminium Oxide Nanoparticles on Allium cepa root tip – Effects of oxidative stress generation and biouptake. Environ Sci Pollut Res. 22(14):11057-11066.

Richardson MA. 2001. Biopharmacologic and herbal therapies for cancer: research update from NCCAM. J Nutr. 131: 3037S-3040S.

Samuelsen AB. 2000. The traditional uses, chemical constituents and biological activities of *Plantago major* L. J Ethnopharmacol. 71(1-2): 1-21.

Samuelsson G. 2004. Drugs of Natural Origin: a Textbook of Pharmacognosy. Stockholm: 5th Swedish Pharmaceutical Press.

Schimmer O, Kruger A, Paulini H, Haefele F. 1994. An evaluation of 55 commercial plant extracts in the Ames mutagenicity test, Pharmazie. 49(6): 448-451.

Smaka-Kincl V, Stegnar P, Lovka M, Toman MJ. 1996. The evaluation of waste, surface and ground water quality using the *Allium* test procedure. Mutat Res Genet Toxicol. 368(3): 171-179.

Sofradžija A, Hadžiselimović R, Maslić E. 1989. Genotoksičnost pesticida. Sarajevo: Svjetlost.

Sudhakar R, Gowda KN, Venu G. 2001. Mitotic Abnormalities Induced by Silk Dyeing Industry Effluents in the Cells of *Allium* cepa. Cytologia. 66(3): 235-239.

Thome P. 1978. The influence of pasture management on the botanical composition of permanent pasture. Schweiz Landw Monats. 56(5): 125-140.

Thome RG, Santos HB, Santos FV, Silva Oliveira RJ, Camargos LF, Pereira MN, Longatti TR, Souto CM, Franco CS, Schuffner ROA, et al. 2012. Evaluation of healing wound and genotoxicity potentials from extracts hydroalcoholic of *Plantago major* and *Siparuna guianensis*. Exp Biol Med. 237: 1379-1386.

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

Citation: S. Wang, Y. Luo, T. Yang, Y. Zhang, Z. Li, W. Jin, Y. Fang (2019) Genetic diversity of *Rhododendron simsii* Planch. natural populations at different altitudes in Wujiashan Mountain. *Caryologia* 72(3): 41-51. doi: 10.13128/caryologia-760

Published: December 13, 2019

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

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

Genetic diversity of *Rhododendron simsii* Planch. natural populations at different altitudes in Wujiashan Mountain (central China)

Shuzhen Wang, Yanyan Luo, Tao Yang, Yujia Zhang, Zhiliang Li, Weibin Jin, Yuanping Fang*

Hubei Key Laboratory of Economic Forest Germplasm Improvement and Resources Comprehensive Utilization; Hubei Collaborative Innovation Center for the Characteristic Resources Exploitation of Dabie Mountains; College of Life Science, Huanggang Normal University, Huanggang, 438000, Hubei Province, P.R. China
*Correspondence author 3559541179@qq.com or wangshuzhen710@whu.edu.cn

Abstract. Altitude could greatly influence species distribution and even their genetic diversity. However, it is unclear how altitude has affected the genetic diversity and population structure of *Rhododendron simsii* Planch., an dominant forestry species in north temperate forest. In this research, 22 polymorphic EST-SSR markers were utilized to assess the genetic diversity of *R. simsii* population distributed at different altitudes of Wujiashan Mountain, a major peak of Dabie Mountains (central China). Totally, 203 alleles were obtained, and each locus gave out 5 to 19 alleles. High genetic diversity existed, as Nei's gene diversity (*h*) and Shannon's Information index (*I*) ranged from 0.728 to 0.920 and 1.430 to 2.690, with the mean value of 0.821 and 1.916, respectively. In particular, 11.1% of genetic differentiation was maintained between populations, while 88.9% occurred within populations. Moreover, moderate gene flow (2.001) among populations was observed, which could effectively resist genetic drift. The genetic diversity of all these five *R. simsii* populations varied significantly with elevation, basically showing high-low-high pattern with elevation increase. Without human intervention, genetic diversity of *R. simsii* populations might increase with the altitude. At the significance level ($p < 0.05$), negative correlation was found between genetic diversity and attenuation rate of light intensity (r=-0.873). Soil of Wujiashan Mountain was acid (the pH value ranged from 4.33 to 4.70), which was rich in organic matter, available phosphorus, available potassium, and alkali hydrolysable nitrogen, as these soil factors interacted with each other to affect the growth of *R. simsii* population. This research would contribute a lot to the knowledge of evolutionary history of *R. simsii* species and benefit subsequent management and conservation actions.

Key words. *Rhododendron simsii* Planch., EST-SSR, genetic diversity, altitude, germplasm protection.

INTRODUCTION

Genetic studies are important for understanding the genetic structure of populations and their ability to respond to natural selection (Lee 2002; Allendorf and Lundquist 2003). Genetic diversity reflects the ability of plant species to adapt environment changes during evolution. Moreover, understanding of genetic diversity in plants, including origin, maintenance and distribution, could give great insight into the modes of speciation, adaptation, as well as population dynamics (Bussell 1999). Genetic composition of a certain species is often influenced by various factors, including the history of introduction, founder effects, life-history characteristics, reproductive method, and even the effect of gene drift (Liu et al. 1998; Ye et al. 2003; Dewalt and Hamrick 2004; Liang et al. 2008). In particular, life-history characteristics, including reproductive method, could affect genetic diversity within- and among- populations (Dewalt and Hamrick 2004). Founder effects and genetic drift could reduce the heterozygosity and increase inter-population differentiation (Liang et al. 2008). Moreover, spatial distribution of genetic structure, reflecting adaptation evolution, environmental changes and natural selection effect, is often closely related to breeding mechanisms of the species (Ishihama et al. 2005).

Genetic and geographical structure in natural populations along elevational gradients are often influenced by life history, ecological traits, and biogeographic history (Quiroga and Premoli 2007; Truong et al. 2007). Elevation, or altitudinal gradient, is an assemblage of environmental variables, which could markedly influence the distribution of population genetic variation (Hahn et al. 2012). Therefore, understanding of current distribution pattern of population genetic diversity and differentiation along altitudinal gradients is vital for conservation and reasonable utilization (Mcmahon et al. 2007).

The *Rhododendron* genus, belonging to Ericaceae family, is widely distributed around the northern hemisphere and presents as different ecological types (Popescu and Kopp 2013). Besides high horticultural and medicinal properties, *Rhododendron* plants play important roles in the stability of ecological system. In particular, *R. simsii* is the dominant species in the community of "Dabie Mountains woods" (central China), (Wang et al. 2017). However, *Rhododendron*-based tourism, habitat fragmentation caused by human activities, as well as changes in ecological environment, all have exerted great influence towards natural *Rhododendron* population (Wang et al. 2017). Therefore, research on genetic diversity and ecological conservation of wild *R. simsii* is essential. However, analysis of genetic diversity and population structure of wild *R. simsii* population is limited, especially the populations located on Dabie Mountains.

Microsatellite, or simple sequence repeats (SSR), is abundant, co-dominant, widely distributed in genomes, highly polymorphic, and easily detectable, which has been widely used in genotype mapping, population structure and genetic diversity analysis (Ambreen et al. 2018; Ukoskit et al. 2018). In particular, SSR marker developed from expressed sequence tags (EST), the EST-SSR, showed more convenience in genetic studies, which has a high transferability to related species (Xu et al. 2018; Zhang et al. 2018). In this research, EST-SSR markers were used to investigate the genetic diversity of *R. simsii* populations at different altitudes in Wujiashan Mountain.

MATERIALS AND METHODS

Description of Wujiashan Mountain and Materials

Wujiashan Mountain (115°46'31.37"-115°50'39.20"E, 31°04'43.20"- 31°07'31.60"N, 3.02×10^4 hm^2), is one of the beautiful spot in Dabie Mountains. According to our field investigation, the constructive species making up the brush and forest were mainly species belonging to the families of Lauraceae, Cornaceae, Leguminosae, Anacardiaceae, Fagaceae, and Caprifoliaceae. Fresh leaves of *R. simsii* were collected at different altitudes on Wujiashan Mountain in August 2017 (Table 1). Particularly, the minimum interval between individuals was set as 100m.

Development of EST-SSR markers

Transcriptome data (SRP099282) of *R. simsii* flower tissue was used for the development of EST-SSR markers

Table 1 The location of *R. simsii* populations studied sampled from Wujiashan Mountain.

Population code	Sampling altitudes	Number of individuals	Longitude (E)	Latitude (N)	Percentage of polymorphic loci
1	972m	15	115°47'18"	31°06'53"	100%
2	1,071m	15	115°47'06"	31°06'04"	100%
3	1,167m	15	115°47'01"	31°06'07"	100%
4	1,270m	15	115°46'49"	31°06'08"	100%
5	1,370m	15	115°46'40"	31°06'08"	100%

with MicroSAtellite (MISA, http://pgrc.ipk- gatersleben.de/misa). These SSR-containing unigenes (di-nucleotide units) with sufficient flanking regions (more than 100bp) were chosen for prime pair design with online software Primer 3 (Wang et al. 2010).

DNA Extraction and Genetic diversity analysis based on EST-SSR markers

The modified CTAB (cetyltrimethyl ammonium bromide) method was adopted to extract genomic DNA, which was further diluted to 50ng/μL (Wang et al. 2017). The 10μL PCR amplification system was set, including 5μL 2×*Taq* Plus PCR MasterMix (TianGen, Beijing, China), 0.2 μM for each primer, as well as 50 ng genomic DNA. The PCR amplification conditions included initial denaturation at 95°C for 10 min, followed by 35 amplification cycles (94°C for 30 s, annealing at optimal temperature for 40 s, and 72°C for 50 s), as well as a 7 min elongation step at 72°C. Then, the PCR amplification products were separated on 6% (w/v) denaturing polyacrylamide gels, which were further visualized by silver staining.

Analysis of soil nutrients and attenuation rate of light intensity in sample plots

Five randomized soil cores (3cm in diameter) were taken up from each sampling spot (0-15cm depth), which were dried off in air and sieved through the 1mm sieve according to WiśniowskaKielian and Klima (2010). Available phosphorus and potassium forms were extracted from the soil with lactate reagent according to the Egner-Riehm's method (Sienkiewicz et al. 2011). In particular, the content of available phosphorus (mg/kg of the soil dry matter) were determined through spectrophotometric method using Beckman DU 640 apparatus, while content of available potassium were obtained with atomic absorption spectrometry (AAS) using PU 9100X Philips. Furthermore, contents of alkali hydrolysable nitrogen were determined with alkaline persulfate digestion (Ding et al. 2013). Contents of the soil organic matter (SOM) were calculated with potassium dichromate oxidation method. Soil pH values were measured in 0.01mol/L $CaCl_2$ slurry (1:2.5 soil/solution) using a reference glass electrode (Ding et al. 2013).

The light intensity upon the upper leaf surface and below the bottom leaf of each *R. simsii* plant was measured with luminometer (AS 810), and 50 plant were randomly selected in each sampling pot. The attenuation rate of light intensity was equal to the upper light intensity divided by lower light intensity. STATISTICA (version 6.1, StatSoft) was employed to determine the statistical parameters and correlation coefficients.

Data Analysis

DNA bands were scored for each sample. Population genetic parameters were calculated with POPGENE version 1.31 software, including number of alleles (N_A) per locus, effective number of alleles (N_E) per locus, expected heterozygosity (H_E), observed heterozygosity (H_O), tests for linkage disequilibrium (LD), Nei's (1973) gene diversity (*h*), Shannon's information index (*I*), total-population inbreeding coefficient (F_{IT}), intra-population inbreeding coefficient (F_{IS}), inter-population genetic differentiation coefficient (F_{ST}), gene flow, genetic identify (*GI*), and genetic distance (*D*) between populations (Wu et al. 2011). Moreover, genetic distance matrix among pairs of populations resulting from POPGENE analysis was utilized to create a dendrogram by MEGA software version 4.0. In addition, the correlations between genetic diversity and altitudinal distances, as well as soil factors were tested using DMRT with the software SPSS 17.0. The statistical significance between populations was estimated by two-tailed Student's t test ($P < 0.05$).

RESULTS

Genetic diversity of Rhododendron populations

Among 57 EST-SSR markers, 22 were polymorphic (Table 2), which gave out 203 bands. *R. simsii* had high genetic diversity at species level, and the polymorphic percentage in five populations were all 100%. N_A and N_E ranged from 5 to 19 and 3.674 to 12.437, with the mean value of 9.227 and 6.083, respectively (Table 3). The length of amplified bands ranged from 161 to 268 bp. The average Shannon's information index (*I*) and Nei's gene diversity (*h*) were 1.916 and 0.821, respectively (Table 3). In particular, the highest *I* and *h* was observed at EST-SSR117 locus, while the lowest existed at SSR019 locus (Table 3). Moreover, H_O and H_E ranged from 0.208 to 1.000 and 0.744 to 0.926, with the mean value of 0.862 and 0.828, respectively (Table 3).

The genetic diversity of population was lower than that of the species. At population level, the average N_A and N_E were 5.56 and 4.23, and the mean *I* and *H* were 1.498 and 0.734, respectively (Table 4). The level of genetic variation of these five populations from the highest to lowest revealed by *I* was pop 5> pop 1> pop 3> pop 2> pop 4. In particular, pop 5 gave out

Table 2 Characteristics of SSR primers used in this research. Shown for each primer pair are forward and reverse primer sequences, repeat motif, annealing temperature (Ta), and the size range of alleles fragment (bp).

Locus	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Repeat motif	*Ta* (°C)	Size range (bp)
SSR019	ATCCCATCCCATCTCTCTC	CACAGATGAGAGAAGAGAGC	(CT)25	55	202-212
SSR025	TCGTGTTGGGTTTCTATTGT	TCCATCAAACTACCAACACC	(CT)25	55	236-256
SSR031	GCAATCTTTCCTCCCATCTT	CTTCTGAATGGGTGCTACTT	(AG)26	56	233-245
SSR032	GAAACGTGTCTGTTTTCTCC	CTACCCCAATTTCCACTACC	(CT)28	56	207-231
SSR070	TCTTCCGATTCCATCATTCC	TGGGCGTGATTTGGTTATAA	(CT)22	54	179-203
SSR078	TTCCAGTTCCAATTCATCGG	CCCAACAACAATTCCATCAC	(CT)22	56	161-179
SSR081	GCCCTATCCCTCAACTTTAC	GAGGAGCGTGGTTAGTAATT	(TC)21	55	230-252
SSR082	GTATGGGACCTGTGATTTCC	CTCCAACTAGCTACTCCAAC	(AG)24	57	229-243
SSR090	TTGAAGAACACTCAAGTTGC	ACGTAGAACATTGCTTTCCT	(GA)21	56	187-201
SSR093	GGTATCCGGTTTTCATCACT	ATACCCACTAGCAACAGAGA	(GA)23	55	234-248
SSR097	AGAAAACTGGGAGATGTGTC	AGGTGATCATCTTTGCATGT	(CT)21	55	247-267
SSR105	CCCCTCTTTCTCTCTAGGAT	GAGAGAGAAGCCGATTACAG	(TC)22	56	186-200
SSR110	TAACCTGCCAGTGGAATTAC	TCTACGTACGCCATTGAAAT	(CT)22	55	224-234
SSR111	CTGCAGACATGACATGAAAC	TTTGCTTACCACTCCCATTT	(AG)21	55	244-260
SSR113	TATTGTACAGCTCCCCTTTG	CCTCAATGTTCTATCGACGT	(CT)23	56	186-200
SSR114	TATTGTACAGCTCCCCTTTG	GAACATGTTAAAGCGCTTGA	(TC)21	54	171-183
SSR116	ATTGCTTCTGATACCATCCG	TATCAGCTTTCGAGTTGTCC	(TC)21	55	211-223
SSR117	GCTATTCACTCGTCAAATGC	ATTGTGGGAATGAAGGTCTC	(GA)22	55	229-268
SSR123	CCCTTCCTCTTCTCAAATCC	CGTCATTTTCACACACAGAG	(CT)23	54	174-189
SSR125	CTCTCCCAAAATTAGCCGAT	GAATTGGCTGTTGGATGATG	(CT)21	55	234-246
SSR129	TGAAGCTGTTTTAGACTCCC	CATGATGGGAAAGCAAAGTG	(TC)22	55	161-175
SSR130	CCATGACGAACCCTATTGAT	TCCTGATATTCCTTTGCACA	(AG)21	56	235-245

the most alleles (128), while pop 4 produced the least alleles (119), which were all polymorphic (Table 4). The *I* ranged from 1.423 (pop 4) to 1.565 (pop 5), while *h* ranged from 0.705 (pop 4) to 0.762 (pop 1). The mean H_O and H_E ranged from 0.836 (pop 2) to 0.885 (pop 5) and 0.730 (pop 4) to 0.807 (pop 1), respectively. Basically, the genetic diversity of five populations showed a high-low-high variation pattern, as genetic diversity of *R. simsii* populations sampled at high and low altitude was higher than populations collected at middle altitude. Using an unpaired two-tailed Student t-test, the difference between populations was not statistically significant ($p<0.05$)

Genetic differentiation among populations at different altitudes

Significant genetic differentiation presented among these five *R. simsii* populaitons ($P<0.001$). An AMOVA of the distance matrix for all individuals partitioned overall variation into two levels, including 'among species' and 'among populations'. The F_{IS} and F_{IT} values ranged from -0.508 to 0.447 and -0.215 to 0.715, with the mean value of -0.178 and -0.047, respectively (Table 3). The F_{IS} value was negative for all five populations, ranging from -0.223 (pop 4) to -0.136 (pop 3), inferring that relatively high level of outcross occurred within populations (Table 4). F_{ST} value was calculated to be 0.111, suggesting that only 11.1 percent of overall genetic variation occurred between populations, while 88.9 percent took place within populations (Table 3). Furthermore, genetic variation mainly occurred at the SSR019 locus, followed by SSR105, SSR117, and SSR123 loci. In particular, gene flow was 2.001, which occurred frequently at SSR114, SSR097, SSR129, SSR082, and SSR090 loci (Table 3). However, the gene flow was a low-frequency event at SSR019 locus with the *Nm* value of 0.265, inferring that this locus might undergo genetic drift during population evolution (Whitlock and McCauley 1999).

Cluster analysis of different R. simsii populations

Genetic distance between pop 1 and pop 3 was the biggest (D = 0.8169), while their genetic identify was the lowest (GI = 0.4418). However, genetic distance between pop 3 and pop 4 was the smallest (D = 0.3979), while their genetic identify was the highest (GI = 0.6717) (Table 5). Based on the matrix of genetic distance, UPG-

Table 3 Genetic diversity of *R. simsii* populations based on SSR markers, including Number of alleles (N_A), effective number of alleles (N_E), Shannon's information index (I), Nei's gene diversity (h), observed heterozygosity (H_O), expected heterozygosity (H_E), intra-population inbreeding coefficient (F_{IS}), total-population inbreeding coefficient (F_{IT}), inter-population genetic differentiation coefficient (F_{ST}), and gene flow (Nm).

Locus	N_A	N_E	I	h	H_O	H_E	F_{IS}	F_{IT}	Fst	Nm
SSR019	5	3.674	1.430	0.728	0.208	0.744	0.447	0.715	0.486	0.265
SSR025	11	6.751	2.090	0.852	0.964	0.860	-0.286	-0.154	0.103	2.184
SSR031	7	4.018	1.598	0.751	0.900	0.757	-0.278	-0.182	0.075	3.105
SSR032	13	7.904	2.245	0.874	1.000	0.880	-0.243	-0.146	0.078	2.937
SSR070	13	9.047	2.361	0.890	1.000	0.896	-0.225	-0.130	0.078	2.960
SSR078	10	6.468	2.036	0.845	1.000	0.852	-0.338	-0.169	0.126	1.738
SSR081	12	5.787	2.005	0.827	1.000	0.833	-0.356	-0.189	0.123	1.785
SSR082	9	5.842	1.923	0.829	1.000	0.835	-0.269	-0.204	0.052	4.606
SSR090	8	6.630	1.961	0.849	1.000	0.857	-0.247	-0.175	0.058	4.048
SSR093	8	6.512	1.944	0.846	1.000	0.853	-0.276	-0.172	0.082	2.817
SSR097	11	6.054	2.015	0.835	1.000	0.841	-0.254	-0.193	0.049	4.874
SSR105	8	5.678	1.870	0.824	1.000	0.831	-0.508	-0.215	0.195	1.036
SSR110	6	3.947	1.527	0.747	0.561	0.752	0.201	0.251	0.062	3.754
SSR111	11	5.161	1.915	0.806	1.000	0.813	-0.363	-0.205	0.116	1.904
SSR113	8	6.383	1.949	0.843	0.754	0.850	0.019	-0.078	0.060	3.889
SSR114	7	5.070	1.758	0.803	0.732	0.810	0.053	0.097	0.047	5.116
SSR116	7	4.398	1.660	0.773	0.797	0.778	-0.117	-0.045	0.063	3.716
SSR117	19	12.437	2.690	0.920	0.843	0.926	-0.114	0.097	0.189	1.071
SSR123	9	7.861	2.126	0.873	0.968	0.880	-0.332	-0.109	0.168	1.241
SSR125	7	4.350	1.624	0.770	0.712	0.776	-0.016	0.059	0.074	3.133
SSR129	8	5.630	1.886	0.822	0.908	0.829	-0.161	-0.102	0.05	4.708
SSR130	6	4.221	1.538	0.763	0.623	0.769	0.080	0.208	0.139	1.547
Mean	9.227	6.083	1.916	0.821	0.862	0.828	-0.178	-0.047	0.111	2.001
St. Dev	3.146	1.990	0.295	0.050	0.201	0.050				

Table 4 Genetic diversity of *R. simsii* populations at different altitudes.

Population codes	N_A	Mean N_A	*Mean* N_E	I	h	H_O	H_E	F_{IS}
Pop 1	120	5.46±1.57	4.52±1.30	1.551±0.271	0.762±0.065	0.884±0.222	0.807±0.069	-0.153±0.262
Pop 2	121	5.50±1.50	3.97±1.27	1.457±0.321	0.717±0.110	0.836±0.239	0.750±0.114	-0.151±0.293
Pop 3	124	5.64±1.68	4.27±1.35	1.495±0.396	0.727±0.148	0.849±0.249	0.757±0.154	-0.136±0.358
Pop 4	119	5.41±1.71	3.92±1.21	1.423±0.387	0.705±0.154	0.866±0.257	0.730±0.159	-0.223±0.294
Pop 5	128	5.82±1.62	4.50±1.30	1.565±0.291	0.757±0.077	0.885±0.200	0.784±0.079	-0.167±0.260
Mean	122.4	5.56±0.17	4.23±0.28	1.498±0.060	0.734±0.025	-	-	-

MA cluster analysis assigned these five populations into two groups (Figure 1). Group I possessed pop 3, pop 4, and pop 5, while pop 1 and pop 2 were clustered into group II. Group I could be further divided into two subgroups, Ia and Ib. Particularly, Ib consisted of pop 3 and pop 4. Population 3 appeared to be more closer with population 4 than other populations. The dendrogram indicated that *R. simsii* population clustering had obvious region specificity, as the first group included populations sampled at higher altitudes, while the second possessed populations collected at the lower altitudes of Wujiashan Mountain (Figure 1).

Soil nutrients and attenuation rate of light intensity in five sample plots

Contents of available phosphorus and available potassium ranged from 13.696 mg/kg (pop 2) to 20.850

Table 5 Nei's genetic identity (above diagonal) and genetic distance (below diagonal) between different populations.

Pop ID	Pop 1	Pop 2	Pop 3	Pop 4	Pop 5
Pop 1	-	0.5720	0.4418	0.4718	0.4865
Pop 2	0.5587	-	0.6309	0.5660	0.5332
Pop 3	0.8169	0.4605	-	0.6717	0.6194
Pop 4	0.7512	0.5692	0.3979	-	0.6712
Pop 5	0.7206	0.6288	0.4790	0.3987	-

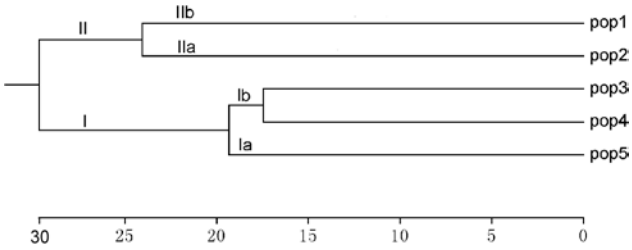


Fig. 1. Dendrogram of five *R. simsii* populations generated with MEAG4 cluster analysis.

mg/kg (pop 5) and 144.378 mg/kg (pop 1) to 306.197 mg/kg (pop 5), with the mean values of 17.329 mg/kg and 204.198 mg/kg, respectively (Figure 2A, 2B and Supplementary file 1). The content of available phosphorus differed slightly between various populations, with a decreasing order of pop 5, pop 3, pop 4, pop 1, and pop 2 (Figure 2A). Moreover, content of soil organic matter in Wujiashan Mountain was 720.953 mg/kg (Figure 2C and Supplementary file 1). Basically, the content of soil organic matter increased with altitude rising, which was highest in pop 5 (838.565mg/kg). Along the elevation, contents of alkali hydrolysable nitrogen were also increased: the lowest value existed in pop 1, while the highest value was observed in pop 5 (Figure 2D). Overall, soil of Wujiashan Mountain was estimated to be rich in nutrients necessary for the growth of *R. simsii*.

The pH value of soil ranged from 4.33 (pop 4) to 4.70 (pop 3), and the mean value was calculated to be 4.494 (Figure 2E). Moreover, the attenuation rate of light intensity in *R. simsii* populations differed significantly, varying from 0.438 to 0.594 (Figure 2F). In particular, pop 5 had the lowest attenuation rate of light intensity (0.438), followed by pop 1 (0.455). However, the highest attenuation rate of light intensity was observed in pop 4 (0.594), followed by pop 3 (0.529).

Correlation between population genetic differentiation and environmental factors

The correlation analysis showed that genetic diversity between populations was not significantly related to altitude ($r_{(I,\ altitude)}$=-0.014, p>0.05; $r_{(h,\ altitude)}$ = -0.136, p>0.05). Moreover, N_A, H_O, H_E, and F_{IS} also showed no relationship with altitude: $r_{(NA,\ altitude)}$=0.599, p>0.05; $r_{(HO,\ altitude)}$=0.599, p>0.05; $r_{(HE,\ altitude)}$=-0.343, p>0.05; $r_{(Fis,\ altitude)}$=-0.474, p>0.05. At the significance level ($p < 0.05$), negative correlation was observed between genetic diversity and attenuation rate of light intensity with r value of -0.873 (Figure 3A). However, no significant correlation were observed between genetic diversity and available phosphorus, available potassium, alkali hydrolysable nitrogen, soil organic matter, as well as pH value of soil at the significance level ($p < 0.05$) by Mantel's test, with the r value ranging from 0.236 to 0.526. In particular, contents of available phosphorus were positively correlated with content of alkali hydrolysable nitrogen (r=0.953, p=0.012) and the content of soil organic matter (r=0.879, p=0.05) (Figure 3B and C). Furthermore, similar correlation also existed between alkali hydrolysable nitrogen content and soil organic matter content (r = 0.935, p = 0.020) (Figure 3D).

Table 6 Contents of available phosphorus, available potassium, alkali hydrolysable nitrogen, soil organic matter, and the pH values of five sampling spots.

Populations	Available phosphorus (mg/kg)	Available potassium (mg/kg)	Soil organic matter (g/kg)	Alkali hydrolysable nitrogen (mg/kg)	pH value	Attenuation rate of light intensity (%)
pop 1	14.578±6.429	144.378±21.666	670.808±39.513	192.356±38.317	4.67	0.455±0.165
pop 2	13.696±3.975	186.313±43.959	658.812±31.972	221.104±43.587	4.43	0.489±0.246
pop 3	20.302±8.97	167.584±10.924	734.673±40.214	327.148±38.510	4.70	0.529±0.229
pop 4	17.220±5.475	216.518±62.862	701.905±37.975	258.014±37.964	4.33	0.594±0.242
pop 5	20.850±2.125	306.197±32.939	838.565±35.178	375.484±42.802	4.34	0.438±0.294
Mean	17.329	204.198	720.953	274.821	4.494	0.501
Standard deviation	3.241	62.838	72.041	75.564	0.179	0.063

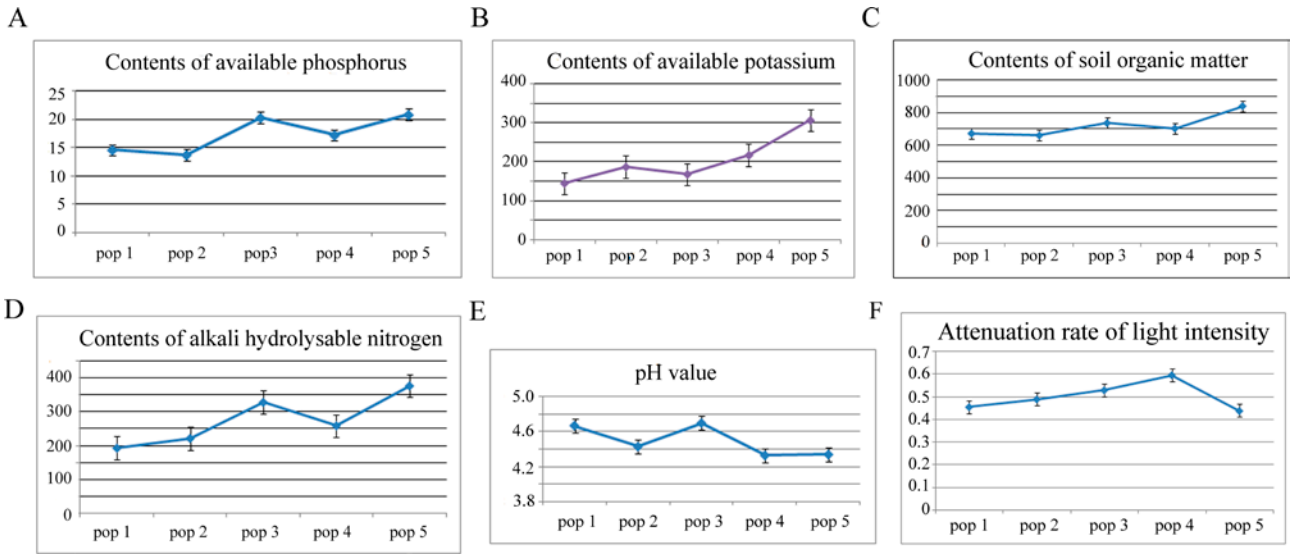


Fig. 2. Contents of aAvailable phosphorus content (A), available potassium content (B), soil organic matter content (C), alkali hydrolysable nitrogen content (D), soil acidity (E), and attenuation rate of light intensity (F) of five *R. simsii* populations. Values were represented as mean value ± standard deviation.

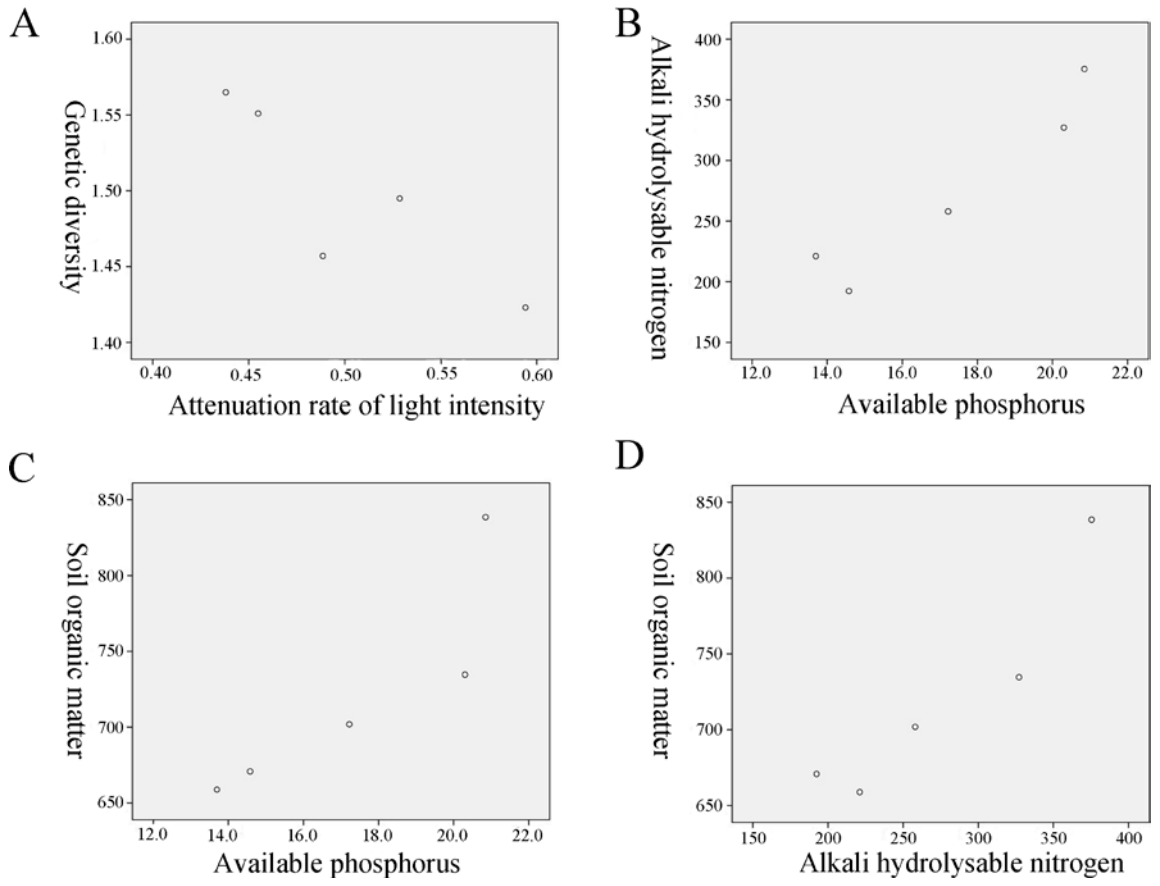


Fig. 3. Correlation between genetic diversity and attenuation rate of light intensity (A), content of alkali hydrolysable nitrogen and available phosphorus (B), soil organic matter and available phosphorus content (C), as well as soil organic matter and alkali hydrolysable nitrogen content (D).

DISCUSSION

Genetic diversity is the result of long-term evolution of a species, which represents the evolutionary potential (Cheng et al. 2017). Moreover, population evolution and the ability to adapt to environment may largely depend on genetic diversity. According to Bussell (1999), deep research on origin, maintenance, as well as distribution of genetic diversity in a species could enhance the understanding of modes of speciation, adaptation, and even population dynamics in the future. *R. simsii*, one of the most valuable woody plants, is dominant shrub with narrow distribution in Dabie Mountains (Li et al. 2015). Global environmental change and travel increase have threatened native biodiversity of wild *R. simsii*, especially the populations located on Dabie Mountain, whose current status need for significant attention.

In this study, high level of genetic diversity was observed in *R. simsii* populations, with *I* and H_E ranging from 1.423 to 1.565 and 0.730 to 0.807, respectively, as H_E ranging from 0.3 to 0.8 means that the tested population possessed high genetic diversity (Frankham et al. 2002; Edwards et al. 2014). The genetic diversity was significantly higher than *Corylus heterophylla* populations in Xingtangsi forest park (*I*=0.4790), *Acer ginnala* sampled at different altitudes in Qiliyu (*I*=0.5070), *Firmiana danxiaensis* located in Danxia landform of China (H_E: 0.364±0.019), *R. decorum* in southwest China (H_E: 0.758±0.048), *Erigeron arisolius* (H_E: 0.748±0.069*),* and *R. jinggangshanicum* population (H_E: 0.642±0.200) sampled from Mount Jinggangshan of China (Yan et al. 2010; Di et al. 2014; Chen et al. 2014; Wang et al. 2013a; Edwards et al. 2014; Li et al. 2015). In our opinion, the ancestor of *R. simsii* located on Wujiashan Mountaian might have a rich genetic basis, which is well preserved during evolution. *R. simsii*, as perennial shrub with overlapping generations, is both wind-pollinated and insect-pollinated plant. Sexual reproduction could increase genetic variation within population, which correspondingly allow natural selection to proceed effectively (Ayres and Ryan 1999; Burt 2000). Therefore, the high genetic diversity existed in *R. simsii* natural populations might be related to the biological characteristics and living conditions. Furthermore, sexual reproduction might be another critical reason for high genetic diversity.

Heterozygote excess was found in this wide *R. simsii* populations (F_{IS} = -0.178), inferring that outcross might occurred, especially in pop 4 (F_{IS} = -0.223) (Nagylaki 1998). Relatively low levels of inbreeding coefficient and outcross also existed in *R. jinggangshanicum* (F_{IS} = 0.023), *R. championiae* (F_{IS} = 0.012), and *R. moulmainense* populations (F_{IS} = 0.045) (Ng and Corlett 2000; Li et al. 2015).Furthermore, 88.9 percent of genetic variation occurred within populations, while only 11.1 percent was maintained between populations (F_{ST} = 0.111, P < 0.001). In particular, genetic variation of *R. simsii* populationswas slightly lower than *R. jinggangshanicum* distributed on Jinggangshan Mountain (93.13%, P < 0.001), but higher than *R. decorum* sampled from Southwest China (85.11%, P < 0.001) and *R. concinnum* collected in Qinling Mountains (85.3%, P < 0.001) (Zhao et al. 2012; Wang et al. 2013b; Li et al. 2015). Gene flow was2.001, higher than *R. arboreum* population (*Nm* = 1.13). Therefore, these *R. simsii* populations might effectively counteract the effect of genetic drift and resist the population differentiation (Kuttapetty et al. 2014). Dendrogram showed typical region specificity, so gene flow might easily occur between neighboring populations.

Genetic diversity of these five *R. simsii* populations varied significantly with elevation (pop 5> pop1>pop 3>pop 2>pop 4), and basically showed high-low-high pattern. In relation to pop 5 with the highest genetic diversity, the contents of available phosphorus, potassium, soil organic matter, and the alkali hydrolysable nitrogen were all the most, while the attenuation rate of light intensity was lowest. During field observation, we found that *R. simsii* population increased basically with altitudes, which reached the maximum at 1,280 meters. According to Leimu et al. (2006), genetic diversity and population size was positively correlated, as well as fitness and population size. Therefore, high genetic diversity at high altitude might be due to the large population size, as effective population size is sufficient to prevent the genetic drift caused by loss of genetic diversity during long-term evolution. Moreover, populations located at 1,280 meters might also possess high level of ecological adapt-ability. Furthermore, the community structure in Wujiashan Mountain had almost no artificial destruction, especially at the high altitude. Local famers plant *R. simsii* as ornamental plant, therefore different genotypes might have been brought to the population at low altitudes. Gene mutation and recombination further enhance the genetic diversity of *R. simsii* populations at low altitudes (Liang et al. 2008).

Soil of Wujiashan Mountain was acid with the pH value ranging from 4.33 to 4.70, and was rich in organic matter, available phosphorus, available potassium, and alkali hydrolysable nitrogen. The typical acid soil is very suitable for the growth of *R. simsii*. Substrate availability could influence microbial metabolic pathways to regulate carbon and even nutrient demand (Mondini et al. 2006). The soil conditions might also exert influence towards the metabolic pathways of microbes associated with *R. simsii*, which further affect the growth of *R.*

simsii population. However, no obvious correlation was observed between these soil factors with genetic diversity of *R. simsii populations*, except the attenuation rate of light intensity.

Relatively high genetic diversity maintained within *R. simsii* populations located on Wujiashan Mountain was observed. No obvious correlation was observed between genetic diversity and altitude. However, genetic diversity was in negative correlation with attenuation rate of light intensity. In particular, the available phosphorus, potassium, soil organic matter, and the alkali hydrolysable nitrogen in soil might interact with each other to affect the growth of *R. simsii* population. This research will be beneficial for the understanding of evolutionary history and population dynamics of *R. simsii* population located on Wujiashan Mountain. In addition, the study is also important for preserving *R. simsii* genetic resources, as well as broadening genetic basis of *Rhododendron* cultivars.

ACKNOWLEDGMENTS

This work was supported by research grants from National Natural Science Foundation of China (NSFC 31500995), Open fund of Hubei Key Laboratory of Economic Forest Germplasm Improvement and Resources Comprehensive Utilization (2017BX06), and Fund of College students' innovative and entrepreneurial activities of Huanggang Normal University.

REFERENCES

Ambreen H, Kumar S, Kumar A, Agarwal M, Jagannath A, Goel S. 2018. Association mapping for important agronomic traits in safflower (*Carthamus tinctorius* L.) core collection using microsatellite markers. Front. Plant Sci. **9**: 402.

Allendorf FW, Lundquist LL. 2003. Introduction: population biology, evolution, and control of invasive species. Conserv. Biol. **17**: 24-30.

Ayres DR, Ryan FJ. 1999. Genetic diversity and structure of the narrow endemic *Wyethia Reticulata* and its congener *W. Bolanderi* (Asteraceae) using RAPD and allozyme techniques. Am. J. Bot. **86**: 344353.

Burt A. 2000. Perspective: sex, recombination, and the efficacy of selection was Weismann right ? Evolution **54**: 337351.

Bussell JD. 1999. The distribution of random amplified polymorphic DNA (RAPD) diversity amongst populations of *Isotoma petraea* (Lobeliaceae). Mol. Ecol. **8**: 775-789.

Chen SF, Li M, Hou RF, Liao WB, Zhou RC, Fan Q. 2014. Low genetic diversity and weak population differentiation in *Firmiana danxiaensis*, a tree species endemic to Danxia landform in northern Guangdong, China. Biochem. Syst. Ecol. **55**: 66-72.

Cheng X, Jiang Y, Tang T, Fan G, Huang X. 2017. Genetic diversity of *Picea likiangensis* natural population at different altitudes revealed by EST-SSR markers. Silvae Genet. **63(5)**: 191-198.

Dewalt SJ, Hamrick JL. 2004. Genetic variation of introduced Hawaiian and native Costa Rican populations of an invasive tropical shrub *Clidemia Hirta* (Melastomataceae). Am. J. Bot. **91**: 1155-1162.

Di XY, Liu KW, Hou SQ, Ji PL, Wang YL. 2014. Genetic variation of hazel (*Corylus heterophylla*) populations at different altitudes in Xingtangsi forest park in huoshan, Shanxi, China. Plant Omics, **7(4)**: 213-220.

Ding X, Han X, Zhang X, Qiao Y. 2013. Effects of contrasting agricultural management on microbial residues in a mollisol in china. Soil Till. Res. **130(6)**: 13-17.

Edwards CE, Lindsay DL, Bailey P, Lance RF. 2014. Patterns of genetic diversity in the rare *Erigeron lemmoni* and comparison with its more widespread congener, *Erigeron arisolius* (Asteraceae). Conserv. Genet. **15**: 419-428.

Frankham R, Ballou JD, Briscoe DA. 2002. Introduction to Conservation Genetics, second edition. New York, Cambridge University Press.

Hahn T, Kettle CJ, Ghazoul J, Frei ER, Matter P, Pluess AR. 2012. Patterns of genetic variation across altitude in three plant species of semi-dry grasslands. PLoS One. **7(8)**: e41608.

Ishihama F, Ueno S, Tsumura Y, Washitani I. 2005. Gene flow and inbreeding depression inferred from fine-scale genetic structure in an endangered heterostylous perennial, *Primula sieboldii*. Mol. Ecol. **14(4)**: 983-990.

Kuttapetty M, Pillai PP, Varghese RJ, Seeni S. 2014. Genetic diversity analysis in disjunct populations of *Rhododendron arboreum* from the temperate and tropical forests of Indian subcontinent corroborate Satpura hypothesis of species migration. Biologia **69(3)**: 311-322.

Lee CE. 2002. Evolutionary genetics of invasive species. Trends. Ecol. Evol. **17**: 386-391.

Leimu R, Mutikainen P, Koricheva J, Fischer M. 2006. How general are positive relationships between plant population size, fitness and genetic variation? J. Ecol. **94**: 942952.

Li M, Chen S, Shi S, Zhang Z, Liao W, Wu W, Zhou R, Fan Q. 2015. High genetic diversity and weak popu-

lation structure of *Rhododendron jinggangshanicum*, a threatened endemic species in Mount Jinggangshan of China. Biochem. Syst. Ecol. **58**: 178-186.

Liang Y, Liu J, Zhang SP, Wang SJ, Guo WH, Wang RQ. 2008. Genetic diversity of the invasive plant coreopsis grandiflora at different altitudes in laoshan mountain, china. Can. J. Plant Sci. **88(4)**: 831-837.

Liu F, Zhang L, Charleswoth D. 1998. Genetic diversity in *leavenworthia* populations with different inbreeding levels. Proc. R. Soc. B. **265**: 293-301.

Mcmahon SM, Harrison SP, Armbruster WS, Bartlein PJ, Beale CM, Meloni ME, Perini MD, Binelli G. 2007. The distribution of genetic variation in Norway spruce (*Picea abies* Karst.) populations in western Alps. J. Biogeogr. **34**: 929-938.

Mondini C, Cayuela ML, Sanchez-Monedero MA, Roig A, Brookes PC. 2006. Soil microbial biomass activation by trace amounts of readily available substrate. Biol. Fert. Soils. **42(6)**: 542-549.

Nagylaki T. 1998. Fixation indices in subdivided populations. Genetics **148**: 1325-1332.

Ng SC, Corlett RT. 2000. Genetic variation and structure in six Rhododendron species (Ericaceae) with contrasting local distribution patterns in Hong Kong, China. Mol. Ecol. **9**: 959-969.

Popescu R, Kopp B. 2013. The genus Rhododendron: An ethnopharmacological and toxicological review. J. Ethnopharmacol. **147**: 42-62.

Quiroga MP, Premoli AC. 2007. Genetic patterns in Podocarpus parlatorei reveal the long-term persistence of cold-tolerant elements in the southern Yungas. J. Biogeogr. **34**: 447-455.

Sienkiewicz S, ŻArczyńSki P, Krzebietke S. 2011. Effect of land use of fields excluded from cultivation on soil content of available nutrients. J. Elementol. **16(1)**: 75-84.

Truong C, Palme AE., Felber F. 2007. Recent invasion of the mountain birch *Betula pubescens* ssp. tortuosa above the treeline due to climate change: genetic and ecological study in northern Sweden. J. Evol. Biol. **20**: 369-380.

Ukoskit K, Posudsavang G, Pongsiripat N, Chatwachirawong P, Klomsa-Ard P, Poomipant P, Tragoonrung S. 2018. Detection and validation of EST-SSR markers associated with sugar-related traits in sugarcane using linkage and association mapping. Genomics. Doi.org/10.1016/j.ygeno.2018.03.019

Wang SZ, Pan L, Hu K, Chen CY, Ding, Y. 2010. Development and characterization of polymorphic microsatellite markers in *Momordica charantia* (Cucurbitaceae). Am. J. Bot. 97(8): e75–e78.

Wang XQ, Huang Y, Long CL. 2013(a) Assessing the genetic consequences of flower-harvesting in *Rhododendron decorum* Franchet (Ericaceae) using microsatellite markers. Biochem. Syst. Ecol. **50**: 296-303.

Wang L, Liao WB, Chen CQ, Fan Q. 2013(b) The seed plant flora of the Mount Jinggangshan region, Southeastern China. PLoS One **8**: e75834.

Wang S, Li Z, Jin W, Xiang F, Xiang J, Fang Y. 2017. Development and characterization of polymorphic microsatellite markers in *Rhododendron simsii* (Ericaceae). Plant Spec. Biol. 32: 100-103.

Whitlock MC, McCauley DE. 1999. Indirect measures of gene flow and migration: Fst≠1/(4Nm+1). Heredity **82**: 117-125.

Wiśniowska-Kielian B, Klima K. 2010. Estimate of available phosphorus and potassium forms content in the winter wheat soils from organic and conventional farms on the background their selected properties. J. Dent. Res. 39(12): 1056-1061.

Wu ZH, Wang SZ, Hu JH, Li F, Ke WD, Ding Y. 2011. Development and characterization of microsatellite markers for *Sagittaria trifolia* var. sinensis (Alismataceae). Am. J. Bot. 98(2): e36-e38.

Xu X, Zhou C, Zhang Y, Zhang W, Gan X, Zhang H, Guo Y, Gan S. 2018. A novel set of 223 EST-SSR markers in Casuarina L. ex Adans.: polymorphisms, cross-species transferability, and utility for commercial clone genotyping. Tree Genet. Genome. **14(2)**: 30.

Yan N, Wang D, Gao YH, Hao XJ, Wang YL. 2010. Genetic diversity of *Acer ginnala* populations at different levation in Qiliyu based on ISSR markers. Sci. Silv. Sin. **46(10)**: 50-56.

Ye WH, Li J, Cao HL, Ge XJ. 2003. Genetic uniformity of *Alternanthera philoxeroides* in South China. Weed Res. **43**: 297-302.

Zhang Z, Xie W, Zhang J, Zhao X, Zhao Y, Wang Y. 2018. Phenotype- and SSR-based estimates of genetic variation between and within two important Elymus species in Western and Northern china. Genes. **9(3)**: 147.

Zhao B, Yin ZF, Xu M, Wang QC. 2012. AFLP analysis of genetic variation in wild populations of five Rhododendron species in Qinling Mountain in China. Biochem. Syst. Ecol. **45**: 198-205.

Supplementary file 1 The correlation coefficient (below) and significant level (above) among genetic diversity, contents of available phosphorus, available potassium, alkali hydrolysable nitrogen, soil organic matter, soil pH value, and the attenuation rate of light intensity.

Item	Genetic diversity	Available phosphorus	Available potassium	Alkali hydrolysable nitrogen	Soil organic matter	pH value	Attenuation rate of light intensity
Genetic diversity	——	0.619	0.703	0.611	0.363	0.614	0.050
Available phosphorus	0.304	——	0.298	0.012	0.05	0.91	0.965
Available potassium	0.236	0.587	——	0.154	0.069	0.127	0.735
Alkali hydrolysable nitrogen	0.311	0.953	0.739	——	0.02	0.696	0.867
Soil organic matter	0.526	0.879	0.849	0.935	——	0.588	0.604
pH value	0.308	-0.071	-0.77	-0.0241	-0.33	——	0.796
Attenuation rate of light intensity	-0.873	0.027	-0.021	-0.105	-0.317	-0.161	——

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: S.I.R. Conceição, A.S. Róis, A.D. Caperta (2019) Nonreduction via meiotic restitution and pollen heterogeneity may explain residual male fertility in triploid marine halophyte *Limonium algarvense* (Plumbaginaceae). *Caryologia* 72(3): 53-62. doi: 10.13128/caryologia-761

Published: December 13, 2019

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

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

Nonreduction via meiotic restitution and pollen heterogeneity may explain residual male fertility in triploid marine halophyte *Limonium algarvense* (Plumbaginaceae)

Sofia I. R. Conceição[1], Ana Sofia Róis[1,2], Ana D. Caperta[1,*]

[1] *Linking Landscape, Environment, Agriculture and Food (LEAF), Instituto Superior de Agronomia (ISA), Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal*
[2] *School of Psychology and Life Sciences, Universidade Lusófona de Humanidades e Tecnologias (ULHT), Campo Grande, 376, 1749-024 Lisboa, Portugal*
* Corresponding author; E-mail address: anadelaunay@isa.ulisboa.pt

Abstract. The cosmopolitan halophylic genus *Limonium* (Plumbaginaceae) presents high cytogenetic interest because of the natural occurrence of diploid and polyploid variants. Natural triploids are very rare in nature but common in this genus, including the widespread triploid *Limonium algarvense* found in the Iberian Peninsula and in Morocco. This study describes male sporogenesis and gametogenesis, pollen formation and germination, and seed production in triploid *L. algarvense* and diploid *Limonium ovalifolium* using various cytological approaches. The diploid species presented regular meiosis. The triploid species was defective in male meiosis due to unpaired chromosomes, trivalent and tetravalent pairing, unbalanced chromosome segregation in meiosis I, and meiotic restitution in both meiosis I and II. These results may be explained by indeterminate and broad first meiotic restitution. Dyads and restitution nuclei at meiosis I were the most frequent meiotic products in the triploid species. Cytomixis was observed in both species, and callose deposition did not differ among them. In the diploid species, regular, tricolpate pollen grains, which germinated in vitro were found. Contrastingly, the triploid species produced heterogeneous pollen in morphology and size, with moderate to no viability that poorly germinated in vitro. We conclude that even if most triploids male gametes are non-functional, they seem to generate small numbers of viable gametes via nonreduction of chromosomes. Flow cytometric seed screening demonstrated that the diploid species presented a diploid progeny whereas triploids only showed triploid progenies. In the triploids low pollen fertility coupled with viable seed production may assure their persistence in natural populations.

Keywords. Apomixis, *In vitro* pollen germination, *Limonium*, Male sporogenesis and gametogenesis, Meiotic restitution, Polyploidy.

INTRODUCTION

In flowering plants, polyploidy (i.e. the condition of having three or more copies of the basic set of chromosomes) has been considered to be one of the

main drivers of plant speciation (Ramsey and Schemske, 1998; Adams and Wendel, 2005). A major route of polyploidization rely on alterations of the meiotic cell cycle involving meiotic nuclear restitution during micro- and megasporogenesis, originating unreduced gametes (Bretagnolle and Thompson, 1995; De Storme and Mason, 2014). Several processes can lead to these gametes like cytokinetic defects, omission of a meiotic division (De Storme and Geelen, 2013) or alterations in spindle biogenesis and polarity. These processes can lead to spindle absence or malformation during metaphase I or II (MI or MII), spindle co-orientation (parallel spindles) in MI (Bretagnolle and Thompson, 1995), and tripolar and fused spindles in MII (Rim and Beuselinck, 1996). Meiotic restitution nuclei can be associated with First Division Restitution (FDR) or Second Division Restitution (SDR) due to the omission of the first or second meiotic division, respectively (Ramanna and Jacobsen, 2003).

The cosmopolitan species-rich genus *Limonium* Mill. (Plumbaginaceae) contains complex aggregates of diploids and polyploids (Róis et al., 2016; Caperta et al., 2017; Róis et al., 2018). These species are of interest because of the occurrence of diploid ($2n$ = 2x = 16, 18 chromosomes), triploid ($2n$ = 3x = 24, 25, 27), tetraploid ($2n$ = 4x = 32, 35, 36), pentaploid ($2n$ = 5x = 43), and hexaploid ($2n$ = 6x = 51, 54, 56) variants (e.g., Erben 1978, 1993; Brullo and Pavone 1981; Arrigoni and Diana 1993; Castro and Rosselló 2007). Meiotic studies in the genus are scarce (D'Amato, 1940a, 1940b, 1949; Róis et al. 2012), although essential to assess fully the nature of cytological variation of polyploid species. Studies on male and female sporogenesis and gametogenesis revealed that diploid *Limonium ovalifolium* (Poir.) Kuntze ($2n$ = 2x =16) presented regular meiosis, whereas tetraploid *Limonium multiflorum* Erben ($2n$ = 4x =35, 36) showed unbalanced and irregular meiosis (Róis et al., 2012; Róis et al., 2016). Triploid cytotypes appear to be the predominant *Limonium* cytotypes in the Iberian Peninsula and in the Balearic Islands (Erben, 1978, 1979; Cowan et al., 1998; Castro and Rosselló, 2007). However, meiotic studies in triploids are limited to a few works on female development in *Statice oleaefolia* Scop. var. *confusa* Godr. (synonym *Limonium virgatum* (Willd.) Fourr.; $2n$ = 27 chromosomes; Erben, 1993) (D'Amato, 1940a, 1940b, 1949).

In the present study, our goal was to compare male sporogenesis and gametogenesis in triploid *Limonium algarvense* Erben with diploid *L. ovalifolium* (Poir.) Kuntze. Both the diploid and triploid species are perennial and capable of vegetative reproduction, and have a widespread occurrence in the Iberian Peninsula and in Morocco (Erben, 1993; Fennane et al., 2014; Caperta et al., 2017). Flowers of the triploid species have potential agro-food industry applications due to antioxidant and anti-inflammatory properties (Rodrigues et al., 2015, 2016).

MATERIAL AND METHODS

Cytological analysis of microsporogenesis and gametogenesis

Five *L. algarvense* ($2n$ = 25 chromosomes, 5.69 ± 0.15 pg/2C; Caperta et al., 2017) and one *L. ovalifolium* ($2n$ = 16, 3.58 ± 0.04 pg/2C; Róis et al., 2012) genotypes from *ex situ* collections established in a greenhouse at Instituto Superior de Agronomia (Lisbon, Portugal) were used.

Microsporogenesis and gametogenesis were analysed in floral buds in distinct developmental stages, as described in Róis et al. (2012). Buds selection was based on size: bud with 0.1 – 0.3 cm (from pre-meiotic interphase to metaphase I - stage I); and bud > 0.3 – 0.5 cm (from anaphase I to pollen grain -stage II). In brief, staged buds were fixed in a fresh absolute ethanol : glacial acetic acid (3:1) solution overnight and stored in 70 % ethanol solution at –20 º C until used. Then, buds were digested in a pectolytic enzyme mixture [2 % cellulase (Sigma), 2 % cellulase "Onozuka R-10" (Serva), and 2 % pectinase enzyme (Sigma)] in 1xEB in a humid chamber for 2 h at 37 °C. Meiocytes, chromosomes and pollen grains spreads were prepared from anthers. Preparations were stained with 4', 6-diamino-2-phenylindole hydrochloride (DAPI) (1 mg ml $^{-1}$) in Vectashield (Vector Laboratories).

The percentage (%) of meiotic products from cell fusion in the first division was calculated as the number of fused dyads/telophase I cells; and the percentage of meiotic products from cell fusion in second division was calculated as the number of fused triads or tetrads/ telophase II cells, respectively.

For tetrad analysis, staged flower buds previously fixed in ethanol : glacial acetic acid (3:1) solution were used. The buds were further placed in an aceto-carmine solution for 1 h, and dissected in a drop of aceto-carmine solution.

For histochemical callose staining, flower buds at stages I and II were collected and stained through a modified procedure by Musiał et al. (2015). Buds were kept in 80 % ethanol for 30 min in agitation and transferred to 1 M NaOH solution for 3 h at 37 ºC. Then, the buds were washed twice in distilled water for 2 min with agitation, and placed in 0.1 M KPO_4 for 2 min at room

temperature (rt), and subsequently in 0.1 % aniline blue (Merck) in 0.1M KPO_4 for 48 h, at rt. Finally, anthers were dissected in multiwall-slides in a drop of 0.1M KPO_4: glycerol (1:1).

Pollen size, viability and germination

Anthers from mature flowers stored in 70 % ethanol were stained using Alexander's stain (Alexander, 1969) under a coverslip, and observed under light microscopy. Total pollen viability estimates were performed by one person using three to five flowers per plant and counted with a 63x objective. About 300 pollen grains per flower were recorded. Pollen grains dimensions were estimated as described in Róis et al. (2012) by calculating mean, standard deviation, and standard error.

For pollen tube growth analysis, five flowers (five anthers each flower) per plant were used following the procedure described in Róis et al. (2012). The pollen grains were collected from plants soon after anther dehiscence and cultured in a media containing 20 mM boric acid, 6 mM calcium nitrate, 0.1 % casein hydrolysate and 7 % sucrose (Zhang et al., 1997). A dialysis tubing and filter paper support combined with 23 % polyethylene glycol –20,000 as an osmoticum in the medium, provided appropriate physical conditions for pollen germination. Pollen grains were incubated at 37 ºC during 48 h or 72 h in the dark. The grains were considered germinated when they present a tube length that was equal or greater than the diameter of the pollen grain. For measurement of pollen tube length, 10 pollen tubes were selected randomly from each treatment, and measured on micrographs recorded with a 63x objective using Axiovision 4.0 (Zeiss) software.

Optical microscopy analysis and imaging

Slides of cell preparations, meiocytes, pollen grains, and pollen tubes were observed using a Zeiss Axioskop 2 fluorescence microscope and photographed with an AxioCam MRc5 digital camera (Zeiss).

Flow cytometric screening of seeds

Flow cytometric seed screening (Matzk et al., 2000) was used to estimate the genome size of seeds derived from each plant progenies in at least 40 seeds, which were analyzed in pooled groups of 20 seeds. Nuclei were isolated following the procedure of Galbraith et al. (1983), in which 0.5 cm^2 of fresh leaf tissue of each sample was chopped with a razor blade, simultaneously with 0.5 cm^2 of fresh leaf tissue of the internal reference standard, in a Petri dish containing 1 ml of WPB buffer (Loureiro et al., 2007). As internal standard *Secale cereale* 'Dankovske' (2C = 16.19 pg) (Doležel et al., 1998) was utilized. The suspension was filtered through a 50 µm mesh nylon filter, and propidium iodide (50 µg/ml) was added to stain the DNA. A Partec CyFlow Space flow cytometer (Partec GmbH, Görlitz, Germany) equipped with a green solid state laser (Cobolt Samba 532 nm, operating at 30 mW; Cobolt, Stockholm, Sweden) was used to measure the relative fluorescence of stained nuclei. Results were obtained using PARTEC FLOMAX software (v. 2.9). About 1300 nuclei per sample were analyzed.

The DNA-ploidy level was inferred as a relative position of the sample G1 peak to that of the internal standard. The exact chromosome numbers and DNA ploidy level of the progenitor plants were determined by chromosome counting (please see Caperta et al., 2017). The value of genome size in mass units (2C in pg; *sensu* Greilhuber et al., 2005) was obtained for each individual analysed using the following equation: *Limonium* 2C nuclear DNA content (pg) = (*Limonium* G_1 peak mean / reference standard G_1 peak mean) * genome size of the reference standard.

Statistics analysis

An analysis of variance (ANOVA) was applied to assess the significance of differences among the studied individuals in relation to meiotic products and pollen types ($p < 0.05$ and $p < 0.001$) (Khan and Rayner, 2003). Percentages were logit-transformed before the statistical analysis to ensure homogeneity of variance.

RESULTS

The diploid *L. ovalifolium* presented a regular meiosis whereas triploid *L. algarvense* showed several meiotic abnormalities (Fig.1). At pre-meiotic interphase, the triploid species exhibited the maximum of three nucleoli while the diploid species showed two nucleoli (data not shown). In the beginning of first meiotic division at the pachytene stage, abnormal meiocytes were observed in *L. algarvense*, with unpaired chromosomes (Fig.1b), although *L. ovalifolium* showed full pairing of all chromosomes (Fig. 1a). In *L. ovalifolium* eight bivalents were found at diakinesis (Fig. 1c) whereas in *L. algarvense*, chromosome abnormalities proceeded as meiosis advanced to the next prophase stages. In this lat-

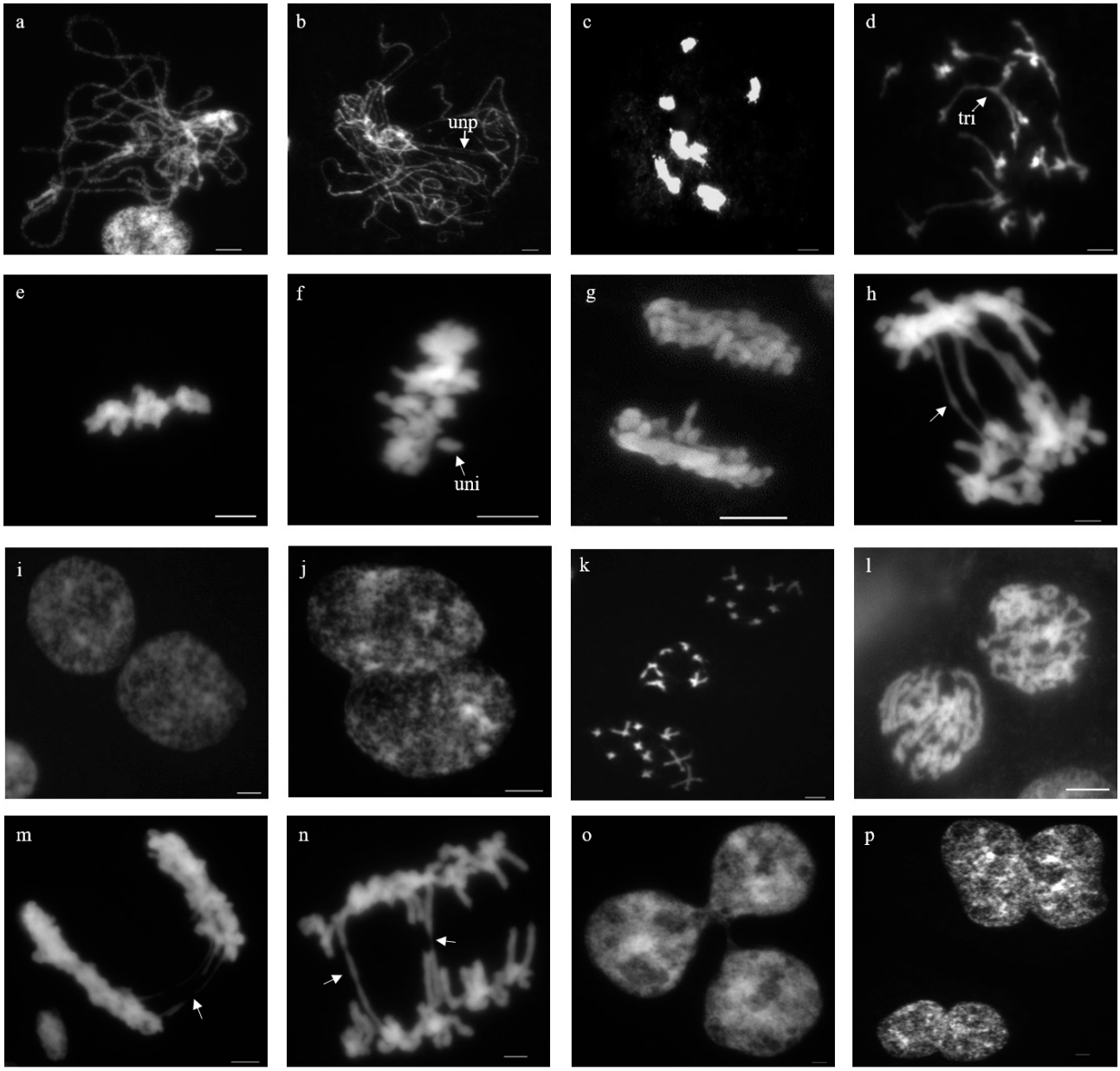


Fig. 1. Chromosome pairing and segregation in DAPI-stained male sporocytes in diploid *Limonium ovalifolium* and triploid *Limonium algarvense*. **a** Full pairing of chromosomes at pachytene in *L. ovalifolium*; **b** Pachytene with unpaired (unp, arrowed) chromosomes in *L. algarvense*; **c** Diakinesis showing eight bivalents in *L. ovalifolium*; **d** Diplotene with trivalents (tri) and different chromosome associations in *L. algarvense*; **e** Metaphase I in *L. ovalifolium*; **f** *L. algarvense* metaphase I showing an univalent (uni, arrowed); **g** Anaphase I in *L. ovalifolium*; **h** Abnormal anaphase I with chromosome bridges in *L. algarvense* (arrowed); **i** Dyad and fused dyads in *L. algarvense* (**j**); **k** Chromosome arrangement after a tripolar spindle in *L. algarvense* showing three groups of chromosomes (10, 6 and 9, respectively totalizing $2n$ = 25 chromosomes); **l** Prophase II in *L. ovalifolium*; **m** Metaphase II and abnormal anaphase II (**n**) showing chromosome bridges in *L. algarvense* (arrowed); **o** Triad fusion in *L. algarvense*; **p** Co-existence of a fused dyad and a fused tetrad in *L. algarvense*. Bars = 5 μm.

ter species, at the diplotene stage trivalents were detected besides bivalent formation (Fig. 1d), and univalents were also visible in metaphase I (Fig. 1f). Conversely, in *L. ovalifolium* metaphase I cells were regular (Fig. 1e). At anaphase I, the majority of *L. ovalifolium* meiocytes were generally normal, although occasionally lagged chromosomes were found (Fig. 1g). By contrast, in *L. algarvense* most anaphase I cells presented chromosome laggards (data not shown) and chromosome bridges (Fig. 1h). At the end of telophase I, some dyads and fused dyads

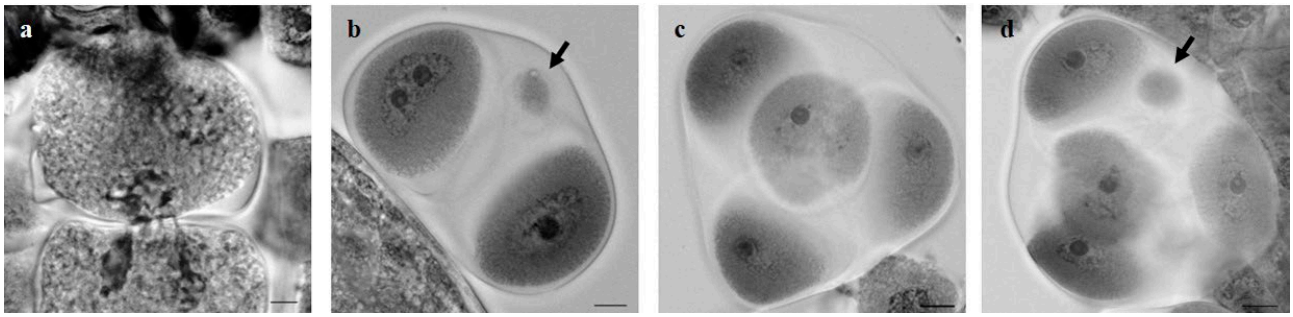


Fig. 2. Tetrad analysis of *L. algarvense*. **a** Cytoplasmatic bridges with chromosome passing (cytomixis); **b** Unbalanced triad (a micronucleus is arrowed); **c** Tetrad; **d** Polyad (a micronucleus is arrowed). Bars = 5 μm.

Table 1. Percentage of meiotic products from *ex-situ* collection *Limonium* plants used.

Species	Accession number	Meiotic Products					1st Division restitution nuclei	2nd Division restitution nuclei	Total of cells analysed
		Monads	Dyads	Triads	Tetrads	Polyads			
L. ovalifolium	2009I4SR	0	10.9	0	83.6	5.5	0	0	65
L. algarvense	2009I1AL	0	23.3	18.5	7.2	0	45.8	5.2	249
	2009I2AL	0	59.6	6.1	0.5	0	28.8	5.1	198
	2009I7AL	10	69.5	5	2.1	0.2	9.4	4.0	479
	2009I18AL	21.9	20.6	1.3	0	0	57.6	2.6	155
	2010I15PA	0	36.9	0	0.2	0.2	57.6	5.2	465

resulting in first division nuclei in the triploid species were found (Fig. 1i-j). However, nuclei fusion was seldom seen in the diploid species. After telophase I in *L. algarvense*, tripolar spindles originate a particular chromosome arrangement where it was possible to detect $2n$ = 25 chromosomes arranged in three groups, respectively ten, six and nine chromosomes (Fig. 1k). In this chromosome arrangement, associations of chromosomes with different sizes and shapes was moreover detected. In meiosis II, regular prophase II cells were observed in *L. ovalifolium* (Fig. 1l). In *L. algarvense* chromosome bridges were still visible in metaphase II and in anaphase II (Fig. 1m, n). At the end of meiosis, the diploid species showed mostly tetrads, while dyads were the more common meiotic product in the triploid species (Table 1). In addition to monads and dyads, the triploid species showed triads and tetrads. The coexistence of fused dyads (Fig. 1p) and tetrads (Fig. 1p) as well as fused triads (Fig. 1o) (second division restitution nuclei) and dyads with micronuclei (Fig.2). Although, the frequency of polyads formation is low in both the diploid and triploid species, its occurrence was rarer in triploid (0.2 %) than in diploid (5.5 %) species (Table 1; Fig. 2d).

In both species, the presence of cytoplasmatic bridges with passage of nuclear content from one cell to another (cytomixis, Fig. 2a) was observed, although with very rare incidence in diploid *L. ovalifolium*. No significant differences were detected between *L. algarvense* individuals in relation to the frequency of the meiotic products types for dyads (P =0.36), triads (P = 0.0692) and tetrads (P = 0.29). No significant differences were found between fused meiotic products for dyads (P =0.49), triads (P = 0.869) and tetrads (P = 0.29).

To substantiate possible causes of nuclei fusion, callose deposition was verified using aniline blue labelling during different stages of microsporogenesis. At tetrad stage both in diploid and triploid plants, bright fluorescence callose labelling was visible around the meiotic products (Fig. 3), without differences between species. The diploid species formed regular tricellular pollen grains with one vegetative nucleus and two sperm nuclei (Fig. 4a) whereas in the triploid species bicellular pollen grains having only one or two vegetative nuclei and one sperm nucleus were observed (Fig. 4b).

In diploid (100%) and triploid species (*L. algarvense* ~ 70%) most pollen grains showed three *colpi* (Fig. 5c, Table 2). Nevertheless, in the triploid species pollen grains with one *colpus*, two, four and five *colpi* were also found (Fig. 5, Table 2). In general, *L. ovalifolium* pollen grains measured 53.52 ± 5.6 μm (n = 41). By contrast, pollen grain size

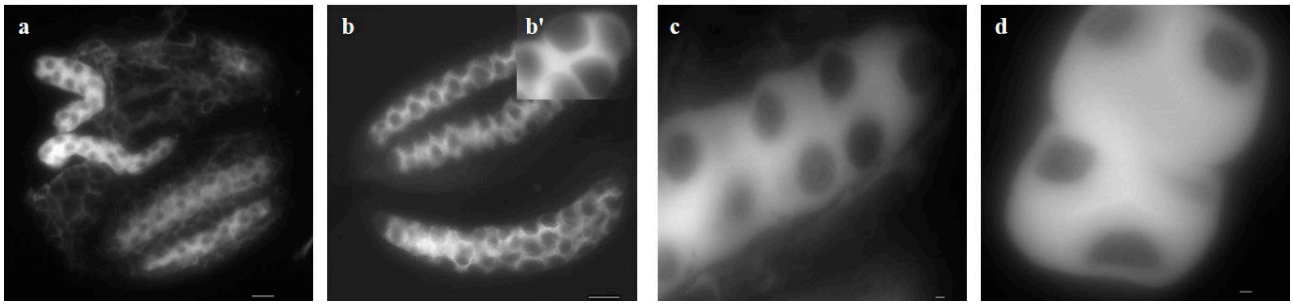


Fig. 3. Callose deposition in *Limonium ovalifolium* and *L. algarvense*. a Anther from a flower bud at stage II with callose labelling in *L. ovalifolium* (bar = 50 μm); **b** Anther from a flower bud at stage II exhibiting bright fluorescence in *L. algarvense* (details shown in the inset – **b'**) (bar = 50 μm)**;** Tetrads with strong labelling in *L. ovalifolium* (**c**) and in *L. algarvense* (**d**) (bar = 5 μm).

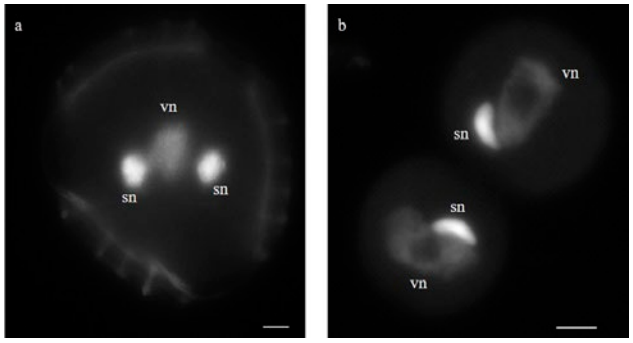


Fig. 4. Dapi staining of pollen grains. **a** Regular tricellular pollen grain in *L. ovalifolium* with one vegetative nucleus and two sperms nuclei; **b** Bicellular polar grain showing one vegetative nucleus (vn) and one sperm nucleus (sn). Bars = 5 μm.

differences were detected in the triploid species: one *colpus* (17.5 ± 2.0 μm, *n* = 2), two (37.8 ± 4.4 μm, *n* = 1), three (56.2 ± 6.3 μm, *n* = 65), four (68.8 ± 3.0 μm, *n* = 51) and five (78.4 ± 1.3 μm, *n* = 9) *colpi*. The pollen grain types did not have significant differences among the studied triploid individuals for 1 *colpus* (*P* = 0.177), 3 *colpi* (*P* = 0.836), 4 *colpi* (*P* = 0.224), 5 *colpi* (*P* = 0.587), and for a significance level between 0.05 and 0.1 for 2 *colpi* (*P* = 0.0545).

Still, pollen grains viability and germination revealed marked differences between species. Comparing to the diploid species, which had 84.8 % (*n* = 1006) of viable grains, the triploid species ranged from low (genotype 2009I1AL, 14.8 %; *n* = 824) to moderate (genotype 2010I15PA, 41.2 %; *n* = 900) viable pollen grains. The diploid species showed the highest frequency (60.5 %, *n* = 885) of germinated grains, while in the triploid species pollen germination frequencies varied among accessions, from 0.8 % (*n* = 900, in 2009I1AL) to 8.2 % (*n* = 883, in 20109I15PA).

The triploid species was able to produce seeds (*c.* 150/per scape) with a moderate seed germination frequency 65 % (*n* = 587). The estimation of embryo and residual endosperm nuclear DNA contents by flow cytometry showed that in *L. ovalifolium* only histograms with a single 2C DNA peak was found representing diploid seeds whereas in *L. algarvense* histograms with an unique 3C DNA peak was detected (Fig. 6).

DISCUSSION

Polyploid plants can arise by the fusion of unreduced gametes or through a mechanism that employs an intermediate step generating triploids (triploid bridge hypothesis) (Ramsey and Schemske, 1998). Triploids are considered to be meiotically unstable, resulting in frequent chromosome loss and fragmentation (McClintock

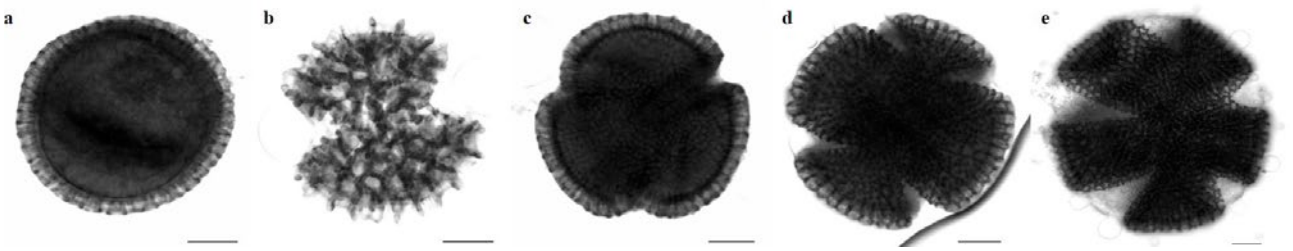


Fig. 5. Pollen grains types in *L. algarvense***.** Pollen grains with one *colpus* (**a**), two (**b**), three (**c**), four (**d**) and five (**e**) *colpi.* Bars = 5 μm.

Table 2. Percentage of pollen grains morphotypes in *L. algarvense*. The percentage of each of the different pollen types and standard deviation is indicated.

Accession	Pollen grains				
	1 *colpus*	2 *colpi*	3 *colpi*	4 *colpi*	5 *colpi*
2009I1AL	0.24 ± 0.2 (2)	1.09 ± 0.5 (9)	74.15 ± 1.8 (611)	23.18 ± 1.8 (191)	1.33 ± 0.7 (11)
2009I7AL	0.61 ±0..3(5)	3.03 ± 1.5 (25)	75.30 ± 3.7 (622)	20.10 ± 2.4 (166)	0.97 ± 0.7(8)
2010I15PA	1.11 ± 0.5 (10)	4.33 ± 2.2(39)	74.11 ±1.5 (667)	19.78 ±3.3(178)	0.67 ± 0.6 (6)
2010I16PA	1.11 ± 1.0 (10)	4.89 ± 1.6 (44)	78.89 ± 5.3 (710)	14.56 ± 7.1 (131)	0.56 ± 0.7 (5)
2009I18AL	0.35 ± 0.3 (3)	1.76 ±1.5 (15)	69.65 ± 6.3 (592)	25.65 ± 3.3 (218)	2.59 ± 2.7 (22)

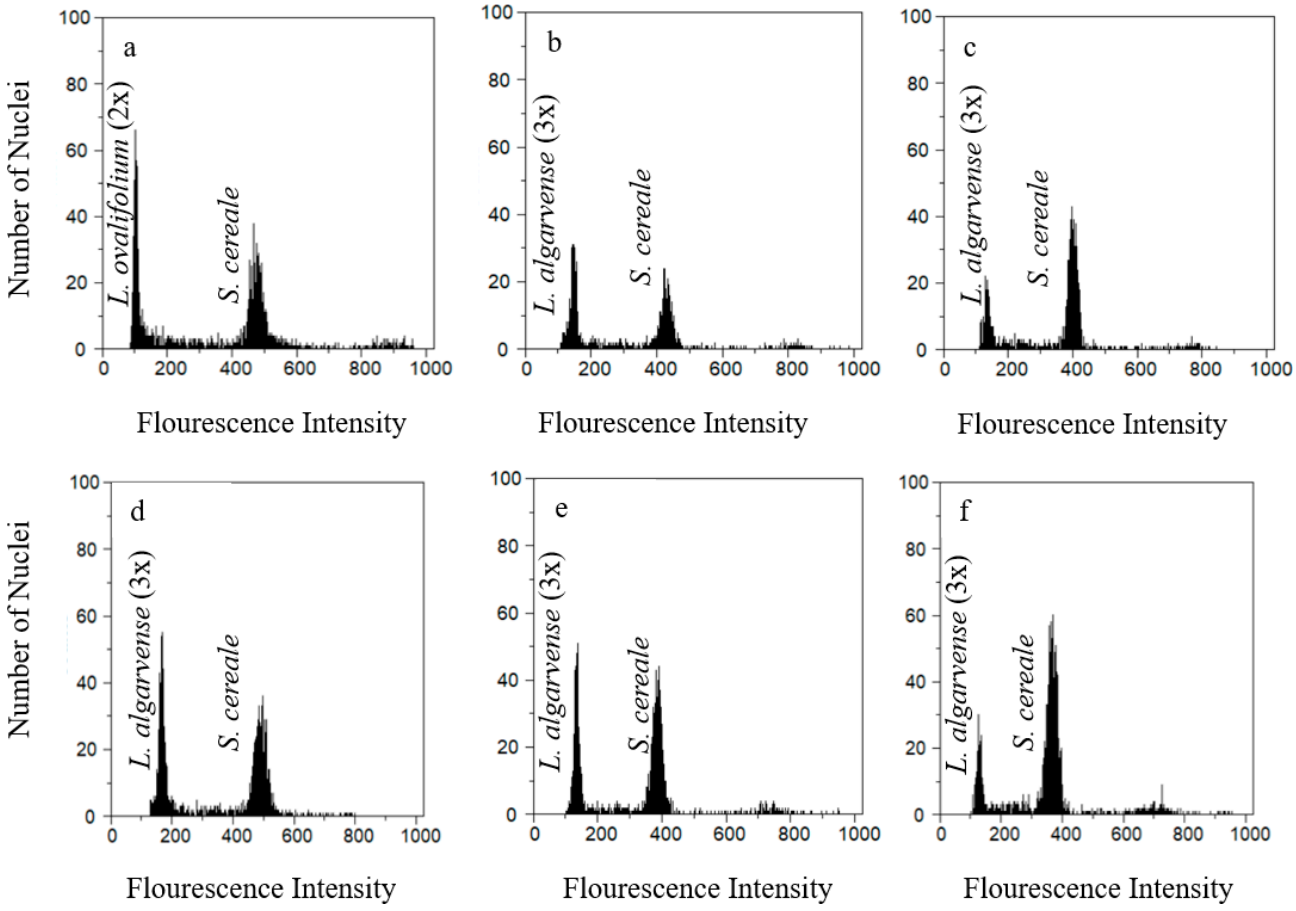


Figure 6. Flow cytometric histograms of seeds of *L. ovalifolium* **(a)** and *L. algarvense* (**b-f**).

1929). These plants can produce diploid, triploid and tetraploid progeny as well as populations of aneuploid individuals with diverse karyotypes (Henry et al., 2005).

During microspore formation, triploid *L. algarvense* showed diverse division anomalies related to chromosome pairing and segregation. In prophase I at pachytene, *L. algarvense* presented unpaired regions, probably as a result of a lack of chromosome homology in some of these regions and reduced recombination. At diplotene and diakinesis the presence of tri- and tetravalents involving non-homologous chromosomes reinforces the hypothesis of intergenomic recombination. Moreover, the tendency for chromosome nondisjunction and a high association of certain groups of chromosomes (with different size and morphology) revealed a high homology between some chromosome regions, which difficult their

normal dissociation during meiosis. Furthermore, the chromosome organization presented in Fig. 1k can be a clue to triploid *L. algarvense* hybrid origin. In this late anaphase I derived from a tripolar fuse, three distinct chromosome groups of six, nine and ten chromosomes were clearly visible, perhaps pointing to three genomes involved in this species formation. This meiotic behaviour strongly suggest parental genome differences for triploid *L. algarvense*, which may imply allopolyploid origin (i.e., attained by hybridization). Distinct genomes usually have several differences at chromosome level as well as modifications in sequence, structure, and/or gene order that difficult or inhibit homologous pairing (Ramsey and Schemske, 2002). These chromosome irregularities during the first meiotic division can be better explained by a broad FDR-type of meiotic restitution (De Storme and Geelen, 2013) or an indeterminate-type meiotic restitution (IMR-type) (Lim et al., 2001). In this latter case, meiotic non-reduction involved a reductional division of bivalents together with an equational segregation of univalents (Lim et al., 2001). Compared to diploid *L. ovalifolium*, triploid *L. algarvense* frequently showed fused nuclei at first division and second division, and dyads, triads, tetrads and polyads. In our study, co-existence of fused dyads and fused tetrads in the same nuclei spread is a strong indication of the occurrence of meiotic restitution. FDR- and/or SDR-type meiotic restitution were considered as important processes for polyploid formation in e.g., *Triticeae* (Jauhar, 2007; Ressurreição et al., 2012), *Solanum* (den Nijs and Peloquin, 1977), *Arachis* (Lavia et al., 2011), and *Taraxacum* (Van Dijk et al., 1999). These processes lead to unreduced gametes formation (Bretagnolle and Thompson, 1995; Brownfield and Köhler, 2011; De Storme and Geelen, 2013).

Callose is an essential barrier between meiocytes and defects on its deposition could lead to an ectopic genome doubling and cell fusion (Spielman et al 1997; Yang et al., 2003; De Storme and Geelen, 2013). Our results showed that nuclear fusion might occur before callose deposition, since throughout anther development its deposition was regular in both species. Another parallel phenomenon that occurred in both species was cytomixis that consists in the movement of the nuclei content between cells (Singhal et al., 2010; Kaur and Singhal, 2012; Mandal et al., 2013), and may lead to unreduced gametes. Although it was not obvious at which stage of meiosis cytomixis occurred, it probably took place at meiosis I before callose deposition. This phenomenon can be one of the precursors of chromatin bridges, micronuclei, triads and polyads, as found in *Spergularia diandra* (Kaur and Singhal, 2012).

Compared to the diploid species that produced regularly sized pollen (Róis et al., 2012), a great diversity of pollen morphology and size was revealed in the triploid species. As previously observed for diploid (*L. ovalifolium*) and tetraploid (*L. multiflorum*) *Limonium* species (Róis et al., 2012), our study supports that pollen size and ploidy are not correlated in the *Limonium* system. Moreover, a direct correlation seems to exist between pollen grain morphology, viability and pollen tube germination, since such processes were only observed in grains predominantly with three *colpi*. In *Limonium*, pollen viability appeared to be high in diploids whereas in polyploids, low to high fertility was reported (Erben, 1978). A high pollen viability was observed in triploid *Turnera sidoides*, which had irregular meiotic behaviour (Kovalsky et al., 2018).

In both diploid and triploid species studied here spontaneous seed production occurred, as insect pollinations were not frequent in our greenhouse. Both species showed a high percentage of seeds per scape, with moderate to high germination. The exact chromosome numbers and DNA ploidy level of the progenitor plants were determined in a previous study by combined flow cytometry and chromosome counting (Caperta et al., 2017). In the present study, flow cytometric seed screening investigations demonstrated that in both species only one DNA peak was found, which corresponds to the embryo peak, since mature seeds were characterized by one embryo and a well-developed starchy endosperm without nuclei (Róis et al., 2012). No quantitative variation in seed ploidy was found in the progeny of diploid or triploid plants and thus *L. ovalifolium* only produced a diploid progeny whereas *L. algarvense* originated a triploid progeny.

CONCLUSIONS

Triploid *L. algarvense* plants displayed extremely unbalanced meiotic cell division, probably originating non-functional aneuploid gametes. However, as found in other natural triploids (Ramsey and Schemske, 1998), these plants may also generate small numbers of euploid (x, 2x) gametes and 3x gametes via non-reduction. Even if the importance of these triploids as pollen donors is limited, they spontaneously produce viable seeds. Although, the reproductive mode of triploid *L. algarvense* is not yet determined, this species show stable populations widespread in the Iberian Peninsula and in Morocco (Caperta et al., 2017), probably originated by apomixis (asexual seed production).

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Ana Paula Paes (LEAF/ISA) for help in plant maintenance and Wanda Viegas for useful comments and suggestions (LEAF/ISA).

FUNDING

This work was funded by Portuguese national funds through Fundação para a Ciência e a Tecnologia (FCT, www.fct.pt/) projects PTDC/AGRPRO/4285/2014 and UID/AGR/04129/2013, grants PTDC/AGRPRO/4285/BM/2014, BPD/UID/AGR/04129/2013 attributed to SIRC and ADC.

REFERENCES

Adams KL, Wendel JF. 2005. Polyploidy and genome evolution in plants. Curr Opin Plant Biol 8(2): 135–141.

Alexander MP. 1969. Differential staining of aborted and nonaborted pollen. Stain Technol 44(3): 117–122

Arrigoni PV, Diana S. 1993. Contribution à la connaissance du genre *Limonium* en Corse. Candollea 48(2): 631–677

Bartolo G, Brullo S, Pavone P. 1981. Números cromosomáticos de plantas occidentales, 138-156. In: Anales del Jardín Botánico de Madrid (Vol. 38, No. 1), Real Jardín Botánico. Madrid, p. 288–299

Bretagnolle F, Thompson JD. 1995. Gametes with the somatic chromosome number: mechanisms of their formation and role in the evolution of autopolyploid plants. New Phytol 129(1): 1–22

Brownfield L, Köhler C. 2010. Unreduced gamete formation in plants: mechanisms and prospects. J Exp Bot 62(5): 1659–1688

Caperta AD, Castro S, Loureiro J, Róis AS, Conceição S, Costa J, Rhazi L, Espírito Santo D, Arsénio, P. 2017. Biogeographical, ecological and ploidy variation in related asexual and sexual *Limonium* taxa (Plumbaginaceae). Bot J Linn Soc 183(1): 75–93.

Castro M, Rosselló JA. 2007. Karyology of *Limonium* (Plumbaginaceae) species from the Balearic Islands and the western Iberian Peninsula. Bot J Linn Soc 155(2): 257–272.

Cowan R, Ingrouille M, Lledó M. 1998. The taxonomic treatment of agamosperms in the genus *Limonium* Mill. (Plumbaginaceae). Folia Geobot 33(3): 353–366.

D'Amato F. 1940. Contributo all'embriologia delle Plumbaginaceae. Nuovo Giorn Bot Ital 47(2): 349–382.

D'Amato F. 1949. Triploidia e apomissia in *Statice oleaefolia* Scop.var. *confusa* Godr. Caryologia 2(2): 71–84.

De Storme N, Geelen D. 2013. Pre-meiotic endomitosis in the cytokinesis-defective tomato mutant *pmcd1* generates tetraploid meiocytes and diploid gametes. J Exp Bot 64(8): 2345–2358.

De Storme N, Mason A. 2014. Plant speciation through chromosome instability and ploidy change: cellular mechanisms, molecular factors and evolutionary relevance. Curr Plant Biol 1: 10–33.

Den Nijs TPM, Peloquin SJ. 1977. Polyploid evolution via 2n gametes. Am Potato J 54(8): 377–386.

Erben M. 1978. Die Gattung *Limonium* im südwestmediterranen Raum. Mitt. Bot. Staatssamml. Münch. 14:361–626

Erben M. 1979. Karyotype differentiation and its consequences in Mediterranean *Limonium*. Webbia 34: 409–417.

Erben M. 1993. *Limonium* Mill. In: Castroviejo S, Aedo C, Cirujano S, Laínz M, Montserrat P, Morales R, Garmendia FM, Navarro C, Paiva J, Soriano C, editors. Flora Iberica ,Vol.3, Real Jardín Botánico, Madrid, CSIC p. 2–143

Fennane M, Ibn-Tattou M, El Oualidi J. 2014. Flore pratique du Maroc. Manuel de détermination des plantes vasculaires, Vol. 3. Travaux de l'Institut Scientifique, Série Botanique No 40. Rabat: Université Mohammed V de Rabat.

Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E. 1983. Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220: 1049-1051.

Greilhuber J, Doležel J, Lysak MA, Bennett MD. 2005. The origin, evolution and proposed stabilization of the terms "genome size' and 'C-value' to describe nuclear DNA contents. Ann. Bot. 95: 255-260.

Henry IM, Dilkes BP, Young K, Watson B, Wu H, Comai L. 2005. Aneuploidy and genetic variation in the *Arabidopsis thaliana* triploid response. Genetics 170(4): 1979–1988.

Jauhar PP. 2007. Meiotic restitution in wheat polyhaploids (amphihaploids): A potent evolutionary force. J Hered 98(2): 188–193.

Kaur D, Singhal VK. 2012. Phenomenon of cytomixis and intraspecific polyploidy (2x, 4x) in *Spergularia diandra* (Guss.) Heldr. & Sart. in the cold desert regions of Kinnaur District (Himachal Pradesh). Cytologia (Tokyo) 77(2): 163–171.

Khan A, Rayner GD. 2003. Robustness to non-normality of common tests for the many-sample location problem. JAMDS 7(4): 187-206.

Kovalsky IE, Luque JMR, Elías G, Fernández SA, Neffa VGS. 2018. The role of triploids in the origin and

evolution of polyploids of *Turnera sidoides* complex (Passifloraceae, Turneroideae). J Plant Res 131(1): 77–89.

Lavia GI, Ortiz AM, Robledo G, Fernández A, Seijo G. 2011. Origin of triploid *Arachis pintoi* (Leguminosae) by autopolyploidy evidenced by FISH and meiotic behaviour. Ann Bot 108(1): 103–111.

Lim KB, Ramanna MS, de Jong JH, Jacobsen E, van Tuyl JM. 2001. Indeterminate meiotic restitution (IMR): a novel type of meiotic nuclear restitution mechanism detected in interspecific lily hybrids by GISH. Theor Appl Genet 103(2-3): 219–230.

Loureiro J, Rodriguez E, Doležel J, Santos C. 2007. Two new nuclear isolation buffers for plant DNA flow cytometry: a test with 37 species. Ann Bot 100: 875–888.

Mandal A, Datta, AK, Gupta S, Paul R., Saha A., Ghosh BK., Bhattacharya A, Iqbal M. 2013. Cytomixis — a unique phenomenon in animal and plant. Protoplasma 250(5): 985–996.

McClintock, B. 1929. A cytological and genetical study of triploid maize. Genetics 14(2): 180–222.

Matzk F, Meister A, Schubert I. 2000. An efficient screen for reproductive pathways using mature seeds of monocots and dicots. Plant J 21(1):97–108.

Musiał K, Kościńska-Pająk M, Antolec R, Joachimiak AJ. 2015. Deposition of callose in young ovules of two *Taraxacum* species varying in the mode of reproduction. Protoplasma 252(1): 135–144.

Ramanna MS, Jacobsen E. 2003. Relevance of sexual polyploidization for crop improvement - A review. Euphytica 133(1): 3–18.

Ramsey J, Schemske DW. 1998. Pathways, mechanisms, and rates of polyploid formation in flowering plants. Annu Rev Ecol Syst 29(1):467–501.

Ramsey J, Schemske DW. 2002. Neopolyploidy in flowering plants. Annu Rev Ecol Syst 33(1): 589–639

Ressurreição F, Barão A, Viegas W, Delgado M. 2012. Haploid independent unreductional meiosis in hexaploid wheat. In: Andrew Swan, editor. Meiosis-molecular mechanisms and cytogenetic diversity. Dublin: Tech Press; p. 321–330.

Rim YW, Beuselinck PR. 1996. Cytology of 2N pollen formation and pollen morphology in diploid *Lotus tenuis* (Fabaceae). Am J Bot 83: 1057–1062.

Rodrigues MJ, Neves V, Martins A, Rauter AP, Neng NR, Nogueira JMF, Varela J, Barreira L, Custódio L. 2016. *In vitro* antioxidant and anti-inflammatory properties of *Limonium algarvense* flowers' infusions and decoctions: A comparison with green tea (*Camellia sinensis*). Food Chem 200: 322–329.

Rodrigues MJ, Soszynski A, Martins A, Rauter AP, Neng NR, Nogueira JMF, Varela J, Barreira L, Custódio L. 2015. Unravelling the antioxidant potential and the phenolic composition of different anatomical organs of the marine halophyte *Limonium algarvens*e. Ind. Crops Prod 77: 315–322.

Róis AS, Teixeira G, Sharbel TF, Fuchs J, Martins S, Espírito-Santo D, Caperta AD. 2012. Male fertility versus sterility, cytotype, and DNA quantitative variation in seed production in diploid and tetraploid sea lavenders (*Limonium* sp., Plumbaginaceae) reveal diversity in reproduction modes. Sexual Pl Reprod 25(4): 305–318.

Róis AS, Sádio F, Paulo OS, Teixeira G, Paes AP, Espírito-Santo D, Sharbel TF, Caperta AD. 2016. Phylogeography and modes of reproduction in diploid and tetraploid halophytes of *Limonium* species (Plumbaginaceae): evidence for a pattern of geographical parthenogenesis. Ann Bot 117(1): 37–50

Róis AS, Castro S, Loureiro J, Sádio F, Rhazi L, Guara-Requena M, Caperta AD. 2018. Genome sizes and phylogenetic relationships suggest recent divergence of closely related species of the *Limonium vulgare* complex (Plumbaginaceae). Plant Syst Evol 304: 955.

Singhal VK, Kaur S, Kumar P. 2010. Aberrant male meiosis, pollen sterility and variable sized pollen grains in *Clematis montana* Buch.-Ham. Ex DC. from Dalhousie hills, Himachal Pradesh. Cytologia 75(1): 31–36.

Spielman M, Preuss D, Li F, Browne WE, Scott RJ, Dickinson HG. 1997. TETRASPORE is required for male meiotic cytokinesis in *Arabidopsis thaliana*. Development 124(13):2645–2657

Van Dijk PJ, Tas ICQ, Falque M, Bakx-Schotman T. 1999. Crosses between sexual and apomictic dandelions (*Taraxacum*). II. The breakdown of apomixis. Heredity 83(6): 715–721.

Yang CY, Spielman M, Coles JP, Li Y, Ghelani, S, Bourdon V, Brown RC, Lemmon BE, Scott RJ, Dickinson HG. 2003. TETRASPORE encodes a kinesin required for male meiotic cytokinesis in *Arabidopsis*. Plant J 34(2): 229–240.

Zhang C, Fountain DW, Morgan ER. 1997. In vitro germination of the trinucleate pollen of *Limonium perezii*. Grana 36(5): 284–288.

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

Citation: Y. Sharafi (2019) Effects of zinc on pollen gamete penetration to pistils in some apple crosses assessed by fluorescence microscopy. *Caryologia* 72(3): 63-73. doi: 10.13128/cayologia-258

Published: December 13, 2019

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

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

Effects of zinc on pollen gamete penetration to pistils in some apple crosses assessed by fluorescence microscopy

Yavar Sharafi

Department of Horticultural sciences, Faculty of Agriculture, Shahed University, Tehran, Iran
E-mail: y.sharafi@shahed.ac.ir

Abstract. Zinc is classically the second abundant moveable metal in plants after iron and represented in all enzyme classes. Zinc generally contributes in the biosynthesis of IAA and GA3 phytohormones which play the major role in fertilization and fruit set. Zinc deficiency leads to reduction in leaf and shoot size, photosynthesis and finally decreases fruit set. Foliar Zinc spray was shown to be efficient and fast for improving Zinc deficiency in fruit trees. In this research the effects of Zinc solution by (0, 3000 and 5000 mg. L-1) were studied on pollen penetration to the pistil and ovary in the four apple cultivars crosses which included "Golden Delicious", "Red Delicious", "Gala" and "Fuji". Spraying was done on the shoots two weeks before blooming in the spring. Pollen penetration was studied using fluorescent microscopy technique 72 and 120 hours after field pollination. Results revealed that the effects of Zinc, crosses and their interaction were significant on pollen germination on the stigma and tube penetration into the primary, middle and beginning of the ovaries and the highest pollen germination on the stigma (43.5%) was observed in the cross (♀Golden Delicious × Gala♂), in 3000 mg. L^{-1} of Zinc 120 hours after pollination and highest pollen tube penetration into the ovary (12/88%) was observed in this cross, respectively. Finally, it was shown that fluorescence microscopy is an accurate technique for nutrition assay in pollination and fruit set. The foliar application of Zinc increased pollen germination and pollen tube growth in all of the crosses.

Keywords. Fruit set, micronutrient, ovary, pollination, pollen tube growth.

INTRODUCTION

Pollen can be used advantageously by breeders, geneticists, physiologists, germplasm supervisors and growers (Dafni and Frimage 2000; Sharafi et al. 2017; Sedgley 1990). In higher plants, pollen grains carry the male gamete on the female part of a flower and play a vital role in breeding program and assist in successful fruit set (Dafni et al. 2012; Hisock and Allen 2008; Sedgley 1990). Generally high crop yield is dependent on viable pollen grains (Dafni et al. 2005). Pollen fertility and viability have dominant prominence in natural and artificial hybridization programs (Sedgley 1990).

Zinc (Zn) is an essential micronutrient in plants and has a vital role in cell division, nucleic acid metabolism, protein synthesis, photosynthesis, carbohydrate metabolism, and phytohormones regulation (Broadley et al. 2007; Chen et al. 2008). Zn directly contributes in the biological synthesis of auxin (IAA) and gibberellin (GA3) which have utmost roles in the plant growth, pollination, fertilization and fruit set in fruit trees (Broadley et al. 2007). Also, Zinc plays a major role as a cofactor in the structure and function of more than 300 enzymes in plants, such as Cu/Zn superoxide (Cu/Zn-SOD), carbonic anhydrase (CA), and sorbitol dehydrogenase (SDH). Zinc toxicity in plants is far less widespread than Zn deficiency (Andreini and Bertini 2012; Moghadam et al. 2013; Nosarszewski et al. 2004; Weinthal et al. 2010). It was reported that about 30% of the agricultural soils in the world show Zn deficiency and Zinc is the most common micronutrient deficient, mostly in high-pH soils (Alloway 2008). Fruit trees which grown in such soils encounter Zn deficiency and show both poor growth and yield quantity and quality. On the other hand, apple (*Malus domestica* L.) commercial cultivars are self- incompatible and therefore, need to be planted along with cross-compatible pollinizer that generates sufficient favorable pollen (Hegedus et al. 2012; Losada and Herrero 2014; Sedgley 1990; Ramirez and Davenpor 2013). On the contrary, apple trees are highly susceptive to Zn deficiency (Alloway 2008). Zn deficiency decreases leaf and shoot size and reduces photosynthetic rates, and thus influences the apple yield and quality (Yan et al. 2010). Zinc deficiency in apple trees is observed as small leaves, late opening of flower and leaf buds, chlorosis between the lateral veins of the leaves, and terminal dieback (Marshner 2011; Macdonal 2000; Sedgley 1990;).

In many fruit trees foliar applications of Zn have been effectively used to promote tree vigor, fruit set, and yield (Wojcik 2007). Some researchers (Golzer and Grant 2006; Qin 1996; Song et al. 2015; Song et al. 2016; Yadav et al. 2013; Zhang et al. 2014) have reported that in the most fruit trees; foliar applications of Zn on mature leaves is unsuccessful and does not provide significant Zn to new leaves produced after spray application or in the following spring. The best time for Zn foliar application is nearly after fruit harvest in the autumn or immediately after pistillate flower senescence followed by two weeks later.

Zhang et al (2013 and 2016) reported that Zinc sulfate spray before bud break increases the activity of carbohydrate metabolic enzymes and regulates endogenous hormone levels in fruits of Fuji apple cultivar. Solar and Stampar (2001) reported that the yield of hazelnut trees was highest in the treatment with 2000 $mg.kg^{-1}$ B + 2000 $mg.L^{-1}$ Zn and lowest in the treatment with 1000 $mg.L^{-1}$ B + 1000 $mg.L^{-1}$ Zn.

Hipps and Davies (2000) reported that foliar application of Zn after blooming could increase the Zn concentration of apple fruit; and spraying Zn on leaves in autumn notably improves the flower Zn content in the coming year. Also, foliar Zn application promotes pollination and cell division.

Moreover, foliar application of Zn was shown to be effective and fast for improving Zn decreasing symptoms in many plants (Sanchez and Righetti 2002). Various studies in palm, citrus and apple showed that foliar application of Zn can significantly increase the Zn concentration, fruit yield and quality (Karimi et al. 2017; Keshavarz et al. 2011; Rodríguez et al. 2005; Neilsen et al. 2004; Neilsen et al. 2005 Khayyat et al. 2007; Zhang et al. 2013). Boron, Iron and Zinc foliar applications have been observed to have a positive effect on chlorophyll contents in B, Fe and Zn deficient plants.

Pollination is one of the most critical stages in the life cycle of a flowering plant, involving a complex series of cell-cell interactions that constitute the pollen-pistil interaction (Dafni et al. 2005; Dafni et al. 2012; Hisock and Allen 2008). In order for fertilization, pollen must first establish molecular compatibility with the stigma and then germinate to produce a pollen tube that penetrates the stigma and grows through the transmitting tissue of the style to locate on the ovule within the ovary (Radonic et al. 2017). Initiation and successful completion of this sequence of events depends upon the stigma and style providing the exact requirements for pollen germination and sustained growth and guidance of the pollen tube through the pistil and ovary. The pollen must therefore be programmed to respond appropriately at every step of this interaction. (Losada and Herrero 2014; Dafni et al. 1998; Nepi and Franchi 2000; Sedgley 1990; Shivanna 2003; Rodriguez-Riano and Dafni 2000). Zn deficiency can have a marked effect on pollination by affecting pollen production, pollen physiology, floral anatomy, and fruit set (Usenik and Stampar 2002).

It has been demonstrated that the first action of stigma is to hook the pollen grains on its surface. For this mechanism receptive stigma must have an adhesive surface. Pollen-stigma interaction is instituted after adhesion of pollen grains on the stigmatic head and multifold incidents occur. The first step is the hydration of the pollen grain and release of wall proteins that bind to receptors on the stigma surface (Radunic et al. 2017; Yellof and Hunt 2005).

Also, florescence microscopy technique accomplished to study pollen tube growth after field and laboratory-controlled pollination and used to identify the

self- and cross-(in) compatibility of cultivars, effective pollination period (EPP), and effects of pollen types on fruit set (Altagic et al. 2012; Kubitscheck 2017). Furthermore, it has not been used for the investigation of the effects of macro and micro nutrients on the pollen germination and tube grow especially in the apple cultivars.

In spite of numerous researches on the response of deficient fruit trees to Zn foliar application, there is no enough information directly appropriate to apple trees. It could be said that because of the five partitions of the apple flower stigma and style; pollen penetration assays in the style have not been used for the screening of nutritional elements on the flower buds (Mularczyk-Oliwa et al. 2017; Sheffield et al. 2005).

The objective of this study was to assess the effects of foliar application of Zn on the apple trees two weeks before bud break in the spring in some crosses by florescence microscopy technique.

MATERIALS AND METHODS

Plant materials, Zn treatments, crosses and pollination

This research was carried out on four apple cultivars which included "Golden Delicious", "Red Delicious", "Gala" and "Fuji". All of the trees were 12-year-old on EM126 rootstocks and foliar sprayed by $ZnSO_4$ as the Zn source (0, 3000 and 5000 mg. L^{-1}) two weeks before bud break in the spring. In the beginning volume of each treatment was calculated based on the fruit trees number and then ZnSO4 was dissolved in distilled water and so sprayed with sprayer on the shoots. Spraying was done in the in the morning (7-8 O clock), when the sky was cloudy and the weather moisture was 70%.

Crosses among the cultivars (six crosses) were programmed as ♀Red delicious × Golden delicious♂, ♀Gala× Fuji♂, ♀Red delicious × Fuji♂, ♀Red delicious × Gala♂, ♀Golden delicious × Fuji♂ and ♀Golden delicious × Gala♂. For each cross four repeats in all direction of the trees were considered and, in each repeat, at least 2 branches with 60 – 100 flower buds were labeled in winter. Selected female cultivar's flower buds were bagged at 'Balloon' stage to prevent the entrance of foreign pollens on the closed pistils. Pollens were collected from the male cultivar flower buds and maintained in freezer until usage in the field pollination time. Pollen germination was tested in an *in vitro* medium before field application on the pistils. Selected female cultivar's flowers were pollinated with selected male parent pollen when the pistils were acceptable for pollens and repeated after 24 hours to increase the pollination accuracy.

Florescence microscopy assessment

Based on the apple trees EPP; 72-120 h is enough for the pollen tubes to reach the ovary hence, pollinated flowers were collected at 72 and 120 h after pollination, sliced and fixed in acid FAA solution (5 % v/v Formaldehyde (40%), 90 % v/v Alcohol ethylic 70% and 5 % Acetic acid glacial 96 %). After rinsing with water two to three times, pistils were cleared in 16% NaOH at room temperature for three days. They were then rinsed in water and stained with 0.1% aniline blue in 0.1% K_2HPO_4. Each part of the pistil was placed on a microscope slide with 10% glycerol and squashed under a glass cover slip. The number of pollen tubes and the rate of pollen tube growth in the different parts of the style were measured using fluorescence microscopy (Olympus AX70). In each pistil the number of germinated pollen grains on the stigma, the number of pollen tubes in the upper and middle parts of the style and in the beginning of the ovary were determined by a fluorescent microscope (Fig 1). Pollen germination percentage was determined by dividing the number of germinated pollen grains by the total number of pollen on the stigma and expressed as a percentage and normalized by angular transformation. The mean of the pollen tube number was calculated as the average number of pollen tubes in different parts of 10 pistils at least. Due to the five partitions of the apple flower stigma and style; the mean of the 5 parts was evaluated for each of them.

Experimental design and data analysis

The experiment was carried out as a factorial based on completely randomized design with three factors

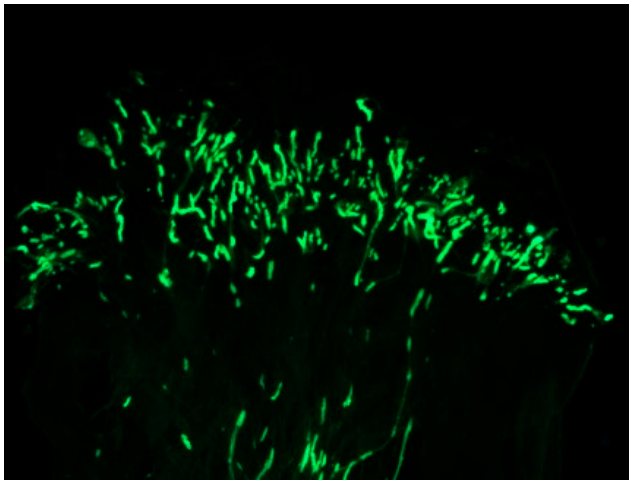


Fig 1. Florescence microscopy photographed from pollens of Red delicious germinated on the Golden delicious stigma and pollen tubes penetrated to the upper part of the style.

including Zn in three levels (0, 3000 and 5000 mg. L^{-1}), time (72 and 120 hrs.' after pollination) and six crosses in five replications (at least ten styles per cross). The data was analyzed using SPSS (24) software. Mean values were analyzed by Duncan's multiple range test.

RESULTS

The analysis of variance showed that the crosses of four cultivars "Golden Delicious", "Red Delicious", "Gala" and "Fuji" had a significant effect on pollen germination percentage on the stigma and pollen tube number which penetrated into the upper and middle parts of the of style and also in the beginning of the ovary at p≤0.01 respectively. Also, Zn concentration and time independently affected the pollen germination percentage on the stigma, the number of pollen tubes which penetrated to the upper and the middle parts of the style and the primary part of the ovary significantly at p≤0.01 (Table 1). The interaction among Zn concentration × crosses was significant on the pollen germination on the stigma, penetration of pollen tubes in the upper and middle parts of the style and so the beginning of the ovaries at p≤0.01 (Table 1), but the interaction of crosses × time was not significant on the studied traits (Table 1).

However, three ways interaction among the time × crosses × Zn concentration was not significant on the pollen germination percentage on the stigma, the number of pollen tubes that penetrated into the middle and the end of the style and the number of pollen tubes in the beginning of the ovary (Table 1).

Based on our findings, the foliar application of Zn on the apple trees enhanced the growth of pollen tubes toward the ovary. In addition, the use of Zn increased the pollen germination on the stigma. About 85 to 90% of pollen germination on the stigma occurred 48 hours after pollination in pollen which was treated by Zn (data not shown). The highest pollen germination percentage on the stigma (43.5%) was observed in the cross (♀Golden Delicious × Gala♂), in 3000 mg. L^{-1} of Zn 120 hours after pollination and the highest pollen tube penetration into the ovary (12/88%) was observed in this cross, respectively. These results may be related to the Zn positive effects on the cell division in pollen tube followed by elongation lead to arrival to the ovaries (Fig. 2).

In comparison with 3000 mg. L^{-1} and 5000 mg. L^{-1} concentration of the Zn, pollen germination rate decreased significantly and showed a toxic effect on pollen. In the crosses which both of pollen parents style parents and were treated by 5000 mg. L^{-1} Zn, pollen germination on the stigma was decreased. This could be related to the toxic effect of Zn. The concentration of 3000 mg l^{-1} of Zn has a positive effect on germination percentage and penetration of pollen tubes, and has been effective in maintaining and integrating the membrane of pollen cells. In this research pollen tubes which penetrated into the style and ovary was also affected. It appeared that the use of Zn on both the male and female parents led to increase in the pollen germination on the stigma in the in-*vivo* condition.

However, in trees treated with 5000 mg. L^{-1} Zn, the number of pollen tubes and the swelling of the tip of the tubes were significantly reduced; this was in accordance with some researchers reports regarding the negative effects of Zn on the vital phenomenon in high concentration (Marschner, 2011; Sedgley, 1990; Sharafi et al., 2017; Zhang et al., 2013; Zhang et al., 2016)

Based on the results of Figure. 1, the highest pollen germination percentage was observed in the cross ♀ Red delicious × Gala ♂ with 43.51% and the highest pollen tube penetration in the cross ♀Red delicious × Golden delicious with 19.27% respectively (Fig. 1).

Table 1. Analysis of variance of the effect of time, crosses and Zn on the pollen germination on the stigma and tube penetration to upper and middle part of the style and so beginning of the ovary.

Beginning of ovary	Middle of style	Upper of style	Stigma	Df	Sources of Variation
9.01 ^{ns}	12.10 ^{ns}	**55.07	**149.64	5	Cross
**149.03	**207.02	**239.06	**4120.07	2	Zn concentration
**765.05	**601.04	**974.05	**2245.44	1	Time
7.01 ^{ns}	15.00 ^{ns}	10.01 ^{ns}	55.97^{ns}	5	Cross × time
**32.04	**02.48	96.50**	83.02 **	10	Cross × Zn
*35.07	*52.06	38.07 *	**327.71	2	Zn ×Time
14.01 ^{ns}	38.07 ^{ns}	19.01 ^{ns}	29.01^{ns}	10	Cross × Zn ×Time
9.04	14.01	13.04	27.03 ^{ns}	144	Error
				179	Total

ns= Non Significant, * = Significant at p ≤0.05, ** = Significant at p ≤0.01.

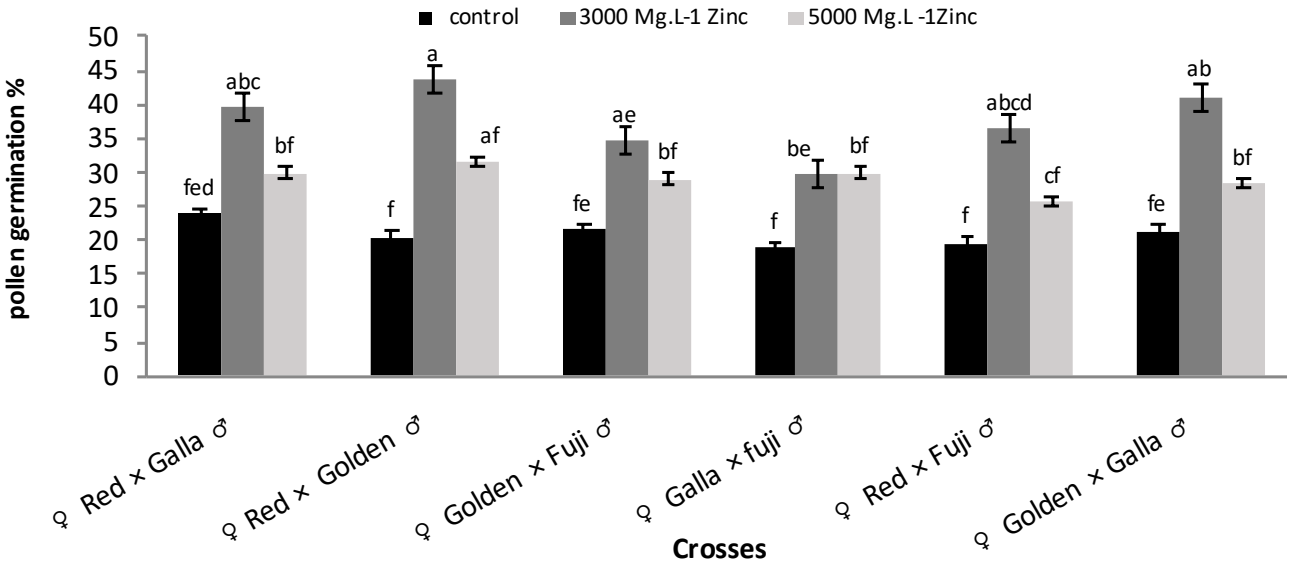


Fig 2. The interaction between Zn concentration and crosses on the pollen germination on the stigma in different crosses. Means in each column, followed by similar letter (s) are not significantly different at 1% probability level.

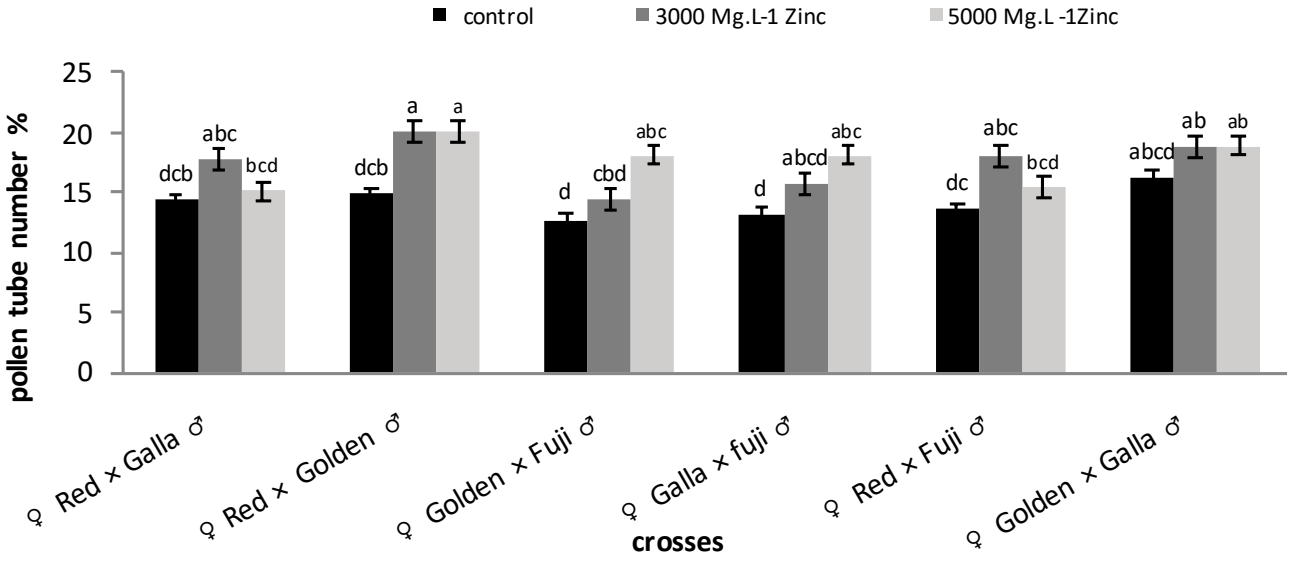


Fig 3. The interactions between Zn concentration and crosses on the pollen tube penetration into the beginning of the style in different crosses. Means in each column, followed by similar letter (s) are not significantly different at 1% probability level.

Results were showed that pollens of Golden delicious on the Red delicious lead to increase the ovule fertilization and finally fruit set among all of the studied crosses (data not shown). The results of Figure 3 showed that the interaction of Zn and crosses significantly affected the pollen tube penetration to the beginning of the styles in all of the six crosses. The highest (20.09%) and lowest (15.2%) pollen tube penetration to the beginning of the styles was observed in the cross ♀ Red delicious × Golden delicious ♂ in the 3000 mg. L^{-1} Zn and not treated crosses (control) respectively and thus, pollen tub penetration to the beginning of the styles was decreased in 5000 mg. L^{-1} Zn while it was higher than the controls in all of the crosses.

It may be connected with the positive effects of Zn on the apple pollen. The positive effects of Zn in high concentration were reported in the most of the plants.

The highest (18.08%) and lowest (13%) pollen tube penetration to the middle part of the styles was observed

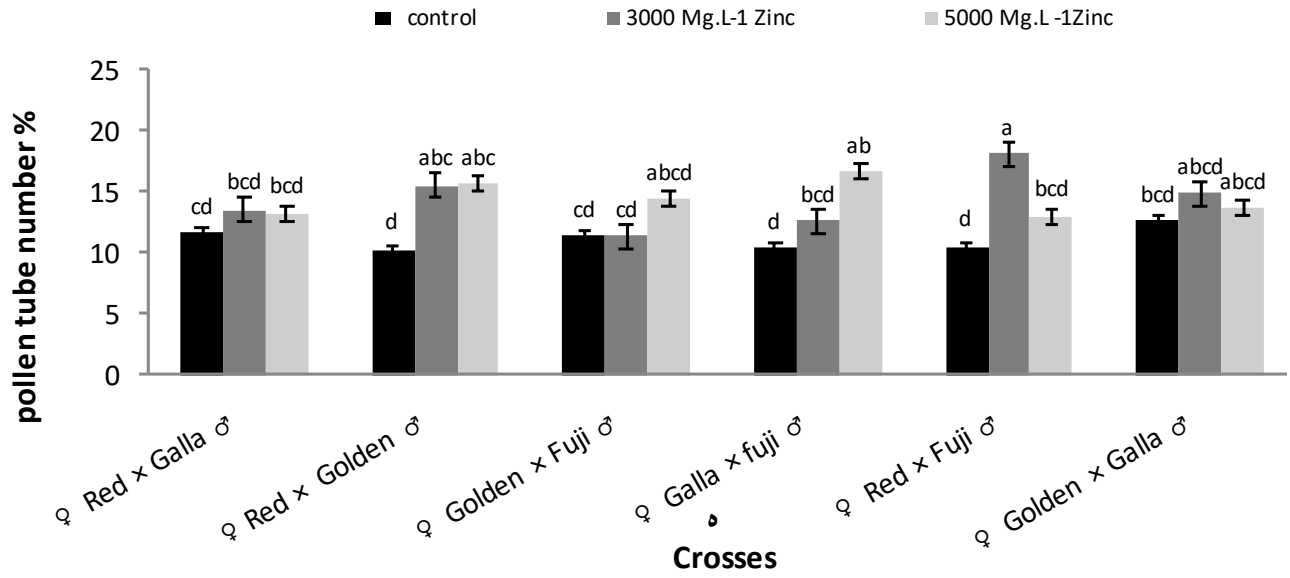


Fig. 4. The interactions between Zn concentration and crosses on the pollen tube penetration into the middle part of the style in different crosses. Means in each column, followed by similar letter (s) are not significantly different at 1% probability level.

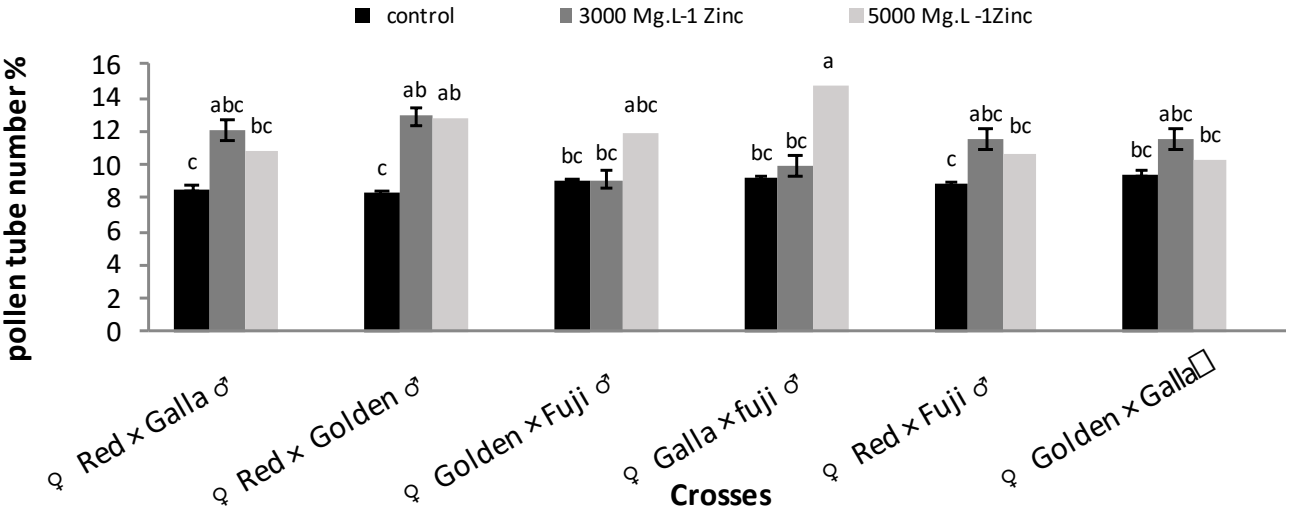


Fig. 5. The interactions between Zn concentration and crosses on the pollen tube penetration into the beginning of the ovary in different crosses. Means in each column, followed by similar letter (s) are not significantly different at 1% probability level.

in the cross ♀ Red delicious × Fuji ♂ in the 3000 mg. L^{-1} Zn and not treated crosses (control) respectively and thus, pollen tube penetration to the middle part of the styles was decreased in 5000 mg. L^{-1} Zn while it was higher than the controls in all of the crosses (Figure 4).

Also, the results of Figure 5 showed that the highest (12.88%) and lowest (10.28%) pollen tube penetration to the middle part of the styles was observed in the cross ♀ Red delicious × Golden delicious ♂ in the 3000 mg. L^{-1} Zn and not treated crosses (control) ♀Golden delicious × Gala ♂ respectively and thus, pollen tub penetration to the middle part of the styles was decreased in 5000 mg. L^{-1} Zn while it was higher than the controls in all of the crosses.

Based on the results shown in Figures 6, 7, 8 and 9 the interaction of Zn concentration and the time after pollination significantly affected the pollen germination on the stigma, tube number in the upper and middle parts of the style and also in the beginning of the ovary in all of the crosses. Maximum (34.28%) and minimum

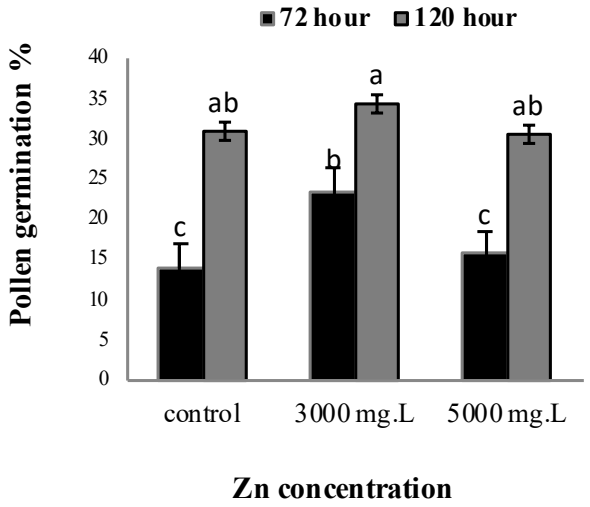


Fig. 6. The interactions between time and Zn concentration on the pollen germination percentage on the stigma. Means in each column, followed by similar letter (s) are not significantly different at 1% probability level.

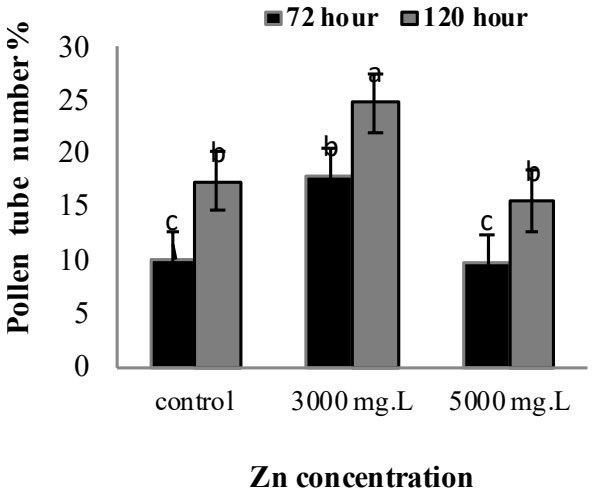


Fig. 7. The interactions between time and Zn concentration on the pollen tube number in the beginning of style. Means in each column, followed by similar letter (s) are not significantly different at 1% probability level.

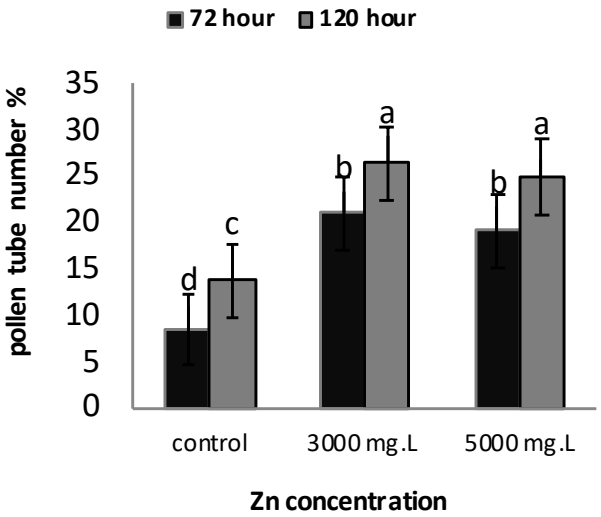


Fig. 8. The interactions between time and Zn concentration on the pollen tube penetration percentage on the middle part of style. Means in each column, followed by similar letter (s) are not significantly different at 1% probability level.

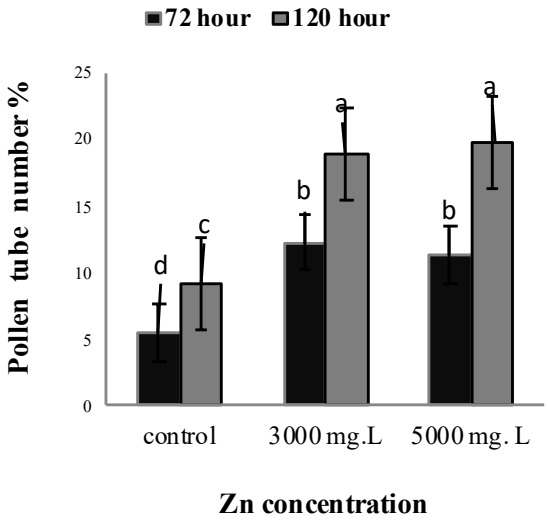


Fig. 9. The interactions between time and Zn concentration on the pollen tube penetration percentage on the beginning of the ovary. Means in each column, followed by similar letter (s) are not significantly different at 1% probability level.

(14.2%) pollen germination on the stigma was observed in the 3000 mg. L^{-1} Zn and not treated crosses (control) respectively and thus, pollen germination on the stigma was decreased in 5000 mg. L^{-1} Zn while it was higher than the controls in all of the crosses (Figure 6).

Maximum (24.73%) and minimum (9.9%) pollen tube penetration to the beginning of the style was observed in the 3000 mg. L^{-1} Zn and not treated crosses (control) respectively and thus, tube penetration to the beginning of the style was decreased in 5000 mg. L^{-1} Zn while it was higher than the controls in all of the crosses (Figure 7).

Maximum (26.48%) and minimum (8.63%) pollen tube penetration to the middle of the style was observed in the 3000 mg. L^{-1} Zn and not treated crosses (control) respectively and thus, tube penetration to the middle of

Table 2. Pearson correlation coefficients for the effect of the Zn on the pollen germination percentage on the stigma and pollen tube penetration to the upper and middle parts of the style and so the beginning of the ovary.

Correlation	Stigma level	Upper of style	Middle of style	Beginning of ovary
Stigma level	1			
Upper of style	0.59^{ns}	1		
Middle of style	0.45^{ns}	**0.79	1	
Beginning of ovary	**0.57	**0.68	0.85**	1

ns= Non Significant, * = Significant at p ≤0.05, ** = Significant at p ≤0.01

the style was decreased in 5000 mg. L^{-1} Zn while it was higher than the controls in all of the crosses (Figure 8).

However, maximum (19.8%) and minimum (5.47%) pollen tube penetration to the beginning of the ovary was observed in the 3000 mg. L^{-1} Zn and not treated crosses (control) respectively and thus, tube penetration to the beginning of the ovary was decreased in 5000 mg. L^{-1} Zn while it was higher than the controls in all of the crosses (Figure 9).

In all of the crosses the highest pollen tube number in the beginning of the style were observed 120 hr after pollination. This phenomenon demonstrated that pollen germination and tube growth were increased followed by the time which may be related to the nutrition case in the style. However, there was a significant difference between the interaction of time and Zn concentration on the pollen tube number in the middle part of the style and in the beginning of the ovary respectively (Figures 8 and 9). Maximum pollen tube numbers in the middle part of the style and in the beginning of the ovary were observed 120 hr after pollination.

Correllation between Zn and pollen germination and penetration to different parts of the style and ovary is showen in Table 2. There was a significant positive correlation between Zn concentrations and germination percentage of pollen on the stigma and the tube number which penetrated to the upper and middle parts of the style and also to the beginning of the ovary respectively (Table 2).The correlation between pollen tubes penetration to the upper (./68) and middle of the style (./85) and the beginning of the ovaries was positive (Table 2).

DISCUSSION

In this research the pollen tube penetration percentage into the styles and ovaries increased by Zn application two weeks before bud break especially in 3000 mg. L^{-1}.

In this study a large number of pollen grains germinated on the stigma exudate and formed callose, indicating good growth of pollen tubes by Zn treatment in apple crosses. However, few of the pollen tubes were observed to penetrate to the style. The average number of pollen tubes was only slightly higher in some crosses. In addition, 120 h after pollination, the average pollen tube length and growth rate were slightly higher in all of the crosses. Previous studies have suggested that self-pollen tubes can grow slower or have higher rates of abrasion than cross-pollen tubes (Golzer and Grant 2006; Qin 1996; Song et al. 2015; Song et al. 2016; Yadav et al. 2013; Zhang et al. 2014; Sedgley 1990).

In accordance with our results Pandey et al, (2006); observed that Zinc is critically required for pollen function and fertilization in lentil. Also, Neilsen et al (2005) observsd that postbloom humic-and fulvic-based zinc sprays can improve apple zinc nutrition also Neilsen et al (2004) reported positive effects of zinc and boron in fertigated high density apple orchards.

Keshavarz et al (2011); reported that foliar application of Zinc and Boron improves walnut vegetative and reproductive growth. They demonstrated the first report of the benefit of foliar B and Zn on pollen germination in walnut trees. There was clear positive effect of B and Zn applied as individual foliar applications and a synergistic effect when applied in combination on walnut yield and quality parameters.

Fei et al (2016); studied enzyme activities, and expression of Zn/Iron-regulated transporter-like protein (ZIP) family genes in the mild, moderate, and severe Zn deficiency in *(Citrus sinensis* L. Osbeck). They reported that the expression of the ZIP family genes, ZIP1, ZIP3, and ZIP4, was promoted by Zn deficiencies. However, chlorophyll contents and net photosynthetic rate decreased with reduction in Zn contents reduction. Also, comparison of severe Zn-deficient and normal leaves revealed increased significant activities of peroxidase (POD) and catalase (CAT, but significantly reduced Zn-containing enzymes such as Cu/Zn superoxide dismutase (Cu/Zn-SOD).

In plants, about half of the ZIP genes could be induced under Zn deficiency, while ZIP1-4 genes seem to be involved in plant Zn transport. Zinc/iron-regulated transporter-like proteins (ZIPs) play a key role for Zn uptake in plants and currently, over 100 ZIP family members have been recognized in diverse plant species (Andreini and Bertini 2012; Moghadam et al. 2013; Nosarszewski et al. 2004; Weinthal et al. 2010).

Furthermore, Zn is involved in various cellular processes, including photosynthesis, nucleic acid and lipid metabolism, protein synthesis, detoxification of reactive oxygen species (ROS), and membrane stability (Broadley et al. 2007). The main role of Zn is involvement in important procedures of cell division and gene expression associated with nucleic acid and protein metabolism. Zinc play an essential role in processes leading to DNA synthesis, and DNA polymerase contains firmly bound zinc. These roles help to explain the nature of some of the symptoms characteristic of Zn deficiency in fruit trees, for example, 'rosetting' and shoot tip dieback (Broadley et al. 2007; Andreini and Bertini 2012; Moghadam et al. 2013; Nosarszewski et al. 2004; Weinthal et al. 2010).

Zinc deficiency leads to malfunctioning of some enzymes, for example, alkaline phosphatase carbonic anhydrase. It could lead to decreased starch formation, accumulation of amino acids, and synthesis of Auxin via impaired tryptophan synthesis. It decreases carbonate dehydrogenase activity which has affected the balance of carbon dioxide and carbon acid and thus has indirectly influenced the rate of photosynthesis. Zinc deficiency has also been found to promote formation of abscisic acid causing premature abscission of leaves and flower buds (Andreini and Bertini 2012; Moghadam et al. 2013; Nosarszewski et al. 2004; Weinthal et al. 2010).

Hence, Zinc is important in DNA and RNA metabolism and protein synthesis and thus, maintains the structural integrity of biomembranes. More than 1,200 protein molecules (Zn metalloprotein) have been identified including a large number of 'zinc-finger'- containing proteins and transcription factors, oxidoreductases and hydrolytic enzymes such as metalloproteases. Furthermore, Zn is a mechanical factor of ribosomes and thus vital for their structural integrity. It plays a major role in carbohydrate metabolism by regulating key enzymes, fructose 1,6-bisphosphatase and aldolase. Synthesis of auxin, and indole acetic acid, is particularly impaired under Zn deficiency (Broadley et al. 2007; Andreini and Bertini 2012; Moghadam et al. 2013; Nosarszewski et al. 2004; Weinthal et al. 2010).

Finally by comparing the effects of the contols with 3000 and 5000 mg. L^{-1} Zn in Figures 1, 2, 3, 4, 5, 6 ,7 and 8 it was demomonstrated that pollen germination and tube penetration to the style and ovary increased until 3000 mg. L^{-1} Zn but, in 5000 mg. L^{-1} mentioned traits were decreased respectively. This could be interpreted that by increasing the Zn concentration it may show toxic effects of pollen tube cell growth. However, in this study, 5000 mg. L^{-1} did not show toxic effect because it showed positive effects on pollen germination and tube penetration to the style and ovary in comparison with the crosses which were not treated with Zn.

CONCLUSION

"It was concluded that fluorescence microscopy technique is a very accurate method for assay of nutrient effects on pollen germination and tube penetration to the pistils in fruit trees in compared with fruit set percentage studies. In this research the mentioned method was used for assay of Zn effects in four apple cultivars crosses which included "Golden Delicious", "Red Delicious", "Gala" and "Fuji". Results showed that the highest pollen germination percentage on the stigma (43.5%) and penetration of pollen tube to the ovary (12/88%) were observed in the cross (♀Golden Delicious × Gala♂), in 3000 mg. L^{-1} of Zn 120 hours after pollination respectively. However, the foliar application of Zn by 1000 mg. L-1 on apple cultivars two weeks before bud break positively affects pollen germination and tube penetration to the ovary in all of the crosses".

ACKNOWLEDGMENT

We would like to acknowledge the research team of Shahed University.

REFERENCES

Alloway BJ. 2004. Zinc in soils and crop nutrition: International Zinc Association Brussels.

Andreini C, Bertini I. 2012. A bioinformatics view of zinc enzymes. J. inorganic biochemistry 111: 150-156.

Atlagić J, Terzić S. Marjanović-Jeromela A. 2012. Staining and fluorescent microscopy methods for pollen viability determination in sunflower and other plant species. J. Industrial crops and products 35 (1): 88-91.

Broadley MR, White PJ, Hammond, JP, Zelko I, Lux A. 2007. Zinc in plants. J. New Phytologist 173 (4): 677-702.

Chen W, Yang X, He Z, Feng Y, Hu F. 2008. Differential changes in photosynthetic capacity, 77 K chlorophyll fluorescence and chloroplast ultrastructure between Zn-efficient and Zn-inefficient rice genotypes (*Oryza sativa*) under low zinc stress. J. Physiologia Plantarum 132 (1): 89-101.

Clément C, Pacini E, Audran JC. 2012. Anther and pollen: from biology to biotechnology: Springer Science & Business Media.

Dafni A, Firmage D. 2000. Pollen viability and longevity: practical, ecological and evolutionary implications. J. Plant Systematics and Evolution 222 (1): 113-132.

Dafni A, Kevan PG, Husband BC. 2005. Practical pollination biology. Practical pollination biology.

Fei, X, Fu X.-z, Wang N-q, XI Jl, Huang Y, Wei Z, Ling L, Peng Lz. 2016. Physiological changes and expression characteristics of ZIP family genes under zinc deficiency in navel orange (*Citrus sinensis*). J. Integrative Agriculture 15 (4): 803-811.

Glozer K, Grant JA. 2006. (101) Effects of Fall Applications of Urea and Zinc Sulfate toBing'Sweet Cherry Spring Budbreak. HortScience 41 (4): 1030-1031.

Hegedűs A, Lénárt J, Halász J. 2012. Sexual incompatibility in Rosaceae fruit tree species: molecular interactions and evolutionary dynamics. Biologia plantarum 56 (2): 201-209.

Hipps N, Davies M. 2000. Effects of foliar zinc applications at different times in the growing season on tissue zinc concentrations, fruit set, yield and grade out of culinary apple trees. Paper read at IV International Symposium on Mineral Nutrition of Deciduous Fruit Crops 564.

Hiscock SJ, Allen AM. 2008. Diverse cell signalling pathways regulate pollen-stigma interactions: the search for consensus. J. New Phytologist 179 (2): 286-317.

Karimi HR, Mohammadi N, Estaji A, Esmaeilizadeh M. 2017. Effect of supplementary pollination using enriched pollen suspension with Zn on fruit set and fruit quality of pistachio. J. Scientia Horticulturae 216: 272-277.

Kubitscheck U. 2017. Fluorescence microscopy: from principles to biological applications: John Wiley & Sons.

Merkusheva MG. 2009. Agrokhimicheskoe mineral'noe syr'e : P, K, S i mikroėlementy. Ulan-Udė: Izd-vo Buriatskogo nauch. tsentra SO RAN.

Moghadam HR, Zahedi TH, Ashkiani A. 2013. Effect of zinc foliar application on auxin and gibberellin hormones and catalase and superoxide dismutase enzyme activity of corn (*Zea mays* L) under water stress. J. Maydica 58 (2013): 218-223.

Mularczyk-Oliwa, M., A. Bombalska, M. Kaliszewski, M. Włodarski, K. Kopczyński, M. Kwaśny, Szpakowska M, Trafny EA. 2012. Comparison of fluorescence spectroscopy and FTIR in differentiation of plant pollens. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 97: 246-254.

Neilsen G, Hogue E, Neilsen D, Bowen P. 2005. Postbloom humic-and fulvic-based zinc sprays can improve apple zinc nutrition. J. HortScience 40 (1): 205-208.

Neilsen G, Neilsen D, Hogue E, Herbert L. 2004. Zinc and boron nutrition management in fertigated high density apple orchards. Canadian J. plant science 84 (3): 823-828.

Nepi M, Franchi G. 2000. Cytochemistry of mature angiosperm pollen. J. Plant Systematics and Evolution 222 (1-4): 45-62.

Nosarszewski M, Clements AM, Downie AB, Archbold DD. 2004. Sorbitol dehydrogenase expression and activity during apple fruit set and early development. J. Physiologia Plantarum 121 (3): 391-398.

Pandey N, Pathak GC, Sharma CP. 2006. Zinc is critically required for pollen function and fertilisation in lentil. J. Trace Elements in Medicine and Biology 20 (2): 89-96.

Pereira MR, Ribeiro H, Cunha M, Abreu I. 2018. Comparison of pollen quality in Vitis vinifera L. cultivars. J. Scientia Horticulturae 227: 112-116.

Phuntsho, Shon SHK, Hong S, Lee S, Vigneswaran S. 2011. A novel low energy fertilizer driven forward osmosis desalination for direct fertigation: evaluating the performance of fertilizer draw solutions. J. Membrane Science 375 (1): 172-181.

Qin X. 1996. Leaf spraying with boron, zinc and magnesium and their effects on the fruit production and quality of Jingchen oranges (Citrus sinensis Osbeck). J. Southwest Agricultural University 18 (1): 40-45.

Radunić M, Jazbec A, Ercisli S, Čmelik Z, Ban SG. 2017. Pollen-pistil interaction influence on the fruit set of sweet cherry. J. Scientia Horticulturae 224: 358-366.

Ramírez, F., and T. L. Davenport. 2013. Apple pollination: a review. J. Scientia Horticulturae 162: 188-203.

Rawashdeh HM, Florin S. 2015. Foliar application with iron as A vital factor of wheat crop growth, yield quantity and quality: A Review. International J. Agricultural Policy and Research 3 (9): 368-376.

Rodríguez VA,. Mazza SM,. Martínez GC,. Ferrero AR. 2005. Zn and K influence in fruit sizes of Valencia orange. J. Revista Brasileira de Fruticultura 27 (1): 132-135.

Sanchez E, Righetti T. 2001. Misleading zinc deficiency diagnoses in pome fruit and inappropriate use of foliar zinc sprays. Paper read at International Symposium on Foliar Nutrition of Perennial Fruit Plants 594.

Sedgley M. 1990. Flowering of deciduous perennial fruit crops. Horticultural Reviews, Volume 12: 223-264.

Sharafi Y, Talebi SF, Talei D. 2017. Effects of heavy metals on male gametes of sweet cherry. J. Caryologia: 1-8.

Sharpley AN. 2000. Agriculture and phosphorus management : the Chesapeake Bay. Boca Raton, Fla.: Lewis Publishers.

Sheffield CS, Smith RF, Kevan PG. 2005. Perfect syncarpy in apple (Malus× domestica 'Summerland McIntosh')

and its implications for pollination, seed distribution and fruit production (Rosaceae: Maloideae). J. Annals of Botany 95 (4): 583-591.

Shivanna K. 2003. Pollen Biology and Biotechnology Science Publishers. Enfield, NH, USA.

Solar A, Stampar F. 2000. Influence of boron and zinc application on flowering and nut set in'Tonda di Giffoni'hazelnut. Paper read at V International Congress on Hazelnut 556.

Song C-Z, Liu M-Y, Meng J-F, Shi P-B, Xi Z-M, Zhang ZW. 2015. Promoting effect of foliage sprayed zinc sulfate on accumulation of sugar and phenolics in berries of Vitis vinifera cv. Merlot growing on zinc deficient soil. J. Molecules 20 (2): 2536-2554.

Song C-Z, Liu M-Y, Meng J-F, Shi P-B, Zhang ZW, Xi Z-M. 2016. Influence of foliage-sprayed zinc sulfate on grape quality and wine aroma characteristics of Merlot. J. European Food Research and Technology 242 (4): 609-623.

Upadhya JC. 1999. Analysis of sulphur, phosphorus and silica in metals, alloys, inorganic compounds and solvents. Mumbai: Bhabha Atomic Research Centre.

Usenik V, Stampar F. 2001. Effect of foliar application of zinc plus boron on sweet cherry fruit set and yield. Paper read at International Symposium on Foliar Nutrition of Perennial Fruit Plants 594.

Weinthal D, Tovkach A, Zeevi V, Tzfira T. 2010. Genome editing in plant cells by zinc finger nucleases. J. Trends in plant science 15 (6): 308-321.

Wojcik P. 2007. Vegetative and reproductive responses of apple trees to zinc fertilization under conditions of acid coarse-textured soil. J. plant nutrition 30 (11): 1791-1802.

Yadav V, Singh P, Yadav P. 2013. Effect of foliar fertilization of boron, zinc and iron on fruit growth and yield of low-chill peach cv. Sharbati. International J. Scientific and Research Publications: 693.

Yan Z, Zhang Y, Zhang Y, Wang Y, F. Zhang, Shu H. 2010. Effects of different zinc application levels on the dynamic accumulation and distribution of dry matter and zinc in apple trees. J. Plant Nutrition and Fertilizer Science 16 (6): 1402-1409.

Yeloff D, Hunt C. 2005. Fluorescence microscopy of pollen and spores: a tool for investigating environmental change. Review of Palaeobotany and Palynology 133 (3): 203-219.

Zhang Y, Fu C, Yan Y, Fan X,. Wang Ya, Li M. 2014. Foliar Application of Sugar Alcohol Zinc Increases Sugar Content in Apple Fruit and Promotes Activity of Metabolic Enzymes. J. HortScience 49 (8): 1067-1070.

Zhang Y, Fu C, Yan Y, Wang Y. a, Li M, Chen M, Qian J, Yang X, Cheng S. 2013. Zinc sulfate and sugar alcohol zinc sprays at critical stages to improve apple fruit quality. J. HortTechnology 23 (4): 490-497.

Zhang Y, Yan Y, Fu. Li CM,. Wang Ya. 2016. Zinc sulfate spray increases activity of carbohydrate metabolic enzymes and regulates endogenous hormone levels in apple fruit. J. Scientia Horticulturae 211: 363-368.

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: N.K. Bhagyanathan, J.E. Thoppil (2019) Active chemical constituents of *Cynanchum viminale* and its cytotoxic effects *via* apoptotic signs on *Allium cepa* root meristematic cells. *Caryologia* 72(3): 75-86. doi: 10.13128/caryologia-769

Published: December 13, 2019

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

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

Active chemical constituents of *Cynanchum viminale* and its cytotoxic effects *via* apoptotic signs on *Allium cepa* root meristematic cells

Neethu Kannan Bhagyanathan*, John Ernest Thoppil

Cell and Molecular Biology Division, Department of Botany, University of Calicut, Kerala, India
*Corresponding author: neethu_dob@uoc.ac.in

Abstract. The present study evaluates the cytotoxic efficacy of methanolic extract of *C. viminale* on *A. cepa* root meristematic cells. DAPI staining was used to study the chromosomal aberrations indued by extract of *C. viminale*. Evans blue staining method was employed to estimate the cell death of root cells of *A. cepa*. The plant extract was found to impart severe cytological damges, specifically chromosomal aberrations at interphase and prophase stage of cell division. Various apoptotic signs such as apoptotic body formation, nuclear budding, micronucleus, nuclear disintegration, nuclear breakage *etc*. were observed in meristematic cells of *A. cepa*. The results suggest the cytotoxic, preferably genotoxic effect of methanolic extract of *C. viminale* as evidenced by various apoptotic symptoms on *A. cepa* root cells.

Keywords. *Cynanchum viminale*, aberrations, *Allium cepa*, cytotoxicity, apoptosis.

INTRODUCTION

Plants have long been used for millennia in traditional medicine against various ailments. Instead of a conventional single compound-single target approach, a consortium of bioactive molecules against multiple targets is gaining more attention nowadays. The synergistic action of various phytochemical compounds acts on various target domains, thus increasing therapeutic efficacy and eliminating the side effects (Cilla et al. 2015). The *Sarcostemma* genus (preferably *Sarcostemma acidum*) is considered as Somalata or Somavalli, also known as moon plant. It is a xerophytic, perennial leafless, jointed trailing shrub with green, cylindrical, fleshy glabrous, twining branches having milky white latex, leaves reduced to scales, opposite, flowers white or pale greenish white. The decoction of the plant is useful to gargle for throat and mouth infection, gonorrhoea, muscle pain *etc*. Recent molecular studies resulted in the taxonomic dissolution of *Sarcostemma* into *Cynanchum* (Meve & Liede- Schumann, 2012).

Allium cepa bioassay is an efficient procedure for assessing chromosome damages induced by plant extracts. It is considered as a preliminary cyto-

toxic screening test which shows high sensitivity and good correlation with mammalian test systems. It is also an important tool for environmental monitoring studies, employed to assess the impacts caused by xenobiotics (Leme & Marin-Morales, 2009; Khanna & Sharma, 2013).

The present study is an attempt to evaluate the phytochemical constituents of methanolic extract of *C. viminale* by GC/MS analysis and its cytotoxic screening with special emphasis on apoptotic signs.

MATERIALS AND METHODS

Plant material

Cynanchum viminale (L.) Bassi (1768: 17) subsp. *viminale* was collected from Karnataka, India (Coordinates: 11.8083° N, 76.6927° E). The specimen was authenticated and a voucher specimen (CALI No. 123742) was deposited at the Herbarium of Department of Botany, University of Calicut, Malappuram, Kerala, India.

Plant extract preparation

10 g of the ground plant materials were subjected to sequential extraction in n-hexane to remove non-polar components followed by 100 mL methanol. The extract thus obtained is then completely evaporated to remove the trace amount of methanol so as to avoid toxicity. Stock solution was prepared in water and different concentrations of plant extracts (200, 400, 600, 800 and 1000 µg/mL) were then made from it.

GC/MS analysis

Chemical composition was determined by GC–MS (Shimadzu QP-2010 Plus with Thermal Desorption System TD 20, fitted with a 60 m × 0.25 mm × 0.25 m WCOT column coated with diethylene glycol (AB-Innowax 7031428, Japan). Helium was used as a carrier gas at a flow rate of 1.21 mL/min at a column pressure of 77.6 kPa. Both injector and detector temperatures were maintained at 260 °C. Samples (6 µL) were injected into the column with a split ratio of 10:0. Component separation was achieved following a linear temperature program of 70-260 °C at 3 ◦C/min and then held at 260 °C for 6 min, with a total run time of 44.98 min. The MS parameters used were: electron ionization (EI) voltage 70 eV, peak width 2 s, mass range 40-850 *m/z* and detector voltage 1.5 V. The constituents were identified by comparison of their linear retention indices. The MS fragmentation pattern was checked with those of other compounds of known composition, with pure compounds and by matching the MS fragmentation patterns with National Institute of Standards and Technology (NIST) mass spectra libraries and with those in the literature (Adams, 2001). Finally, their quantification was performed on the basis of their GC peak areas.

Cytotoxic screening on A. cepa

Prior to initiating the test, the outer dry scales of onion bulbs were removed without destroying the root primordia. They were allowed for rooting by placing in distilled water for 1-2 days. Germinated bulbs with healthy roots (1-2 cm) were collected at a period of maximum mitotic activity (between 9 am and 10 am on sunny days) and washed with distilled water. The bases of bulbs were kept in vials containing different concentrations of plant extracts (200, 400, 600, 800 and 1000 µg/mL) in such a way that only roots were suspended in extracts. Positive and negative controls were also kept *viz*., hydrogen peroxide (2%) and distilled water. Root tips were collected from the different vials at 12 h, 24 h, and 48 h intervals. The collected samples were washed in distilled water and immediately fixed in modified Carnoy's fluid for 1 h. Then the root tips were subjected to hydrolysis with 1N HCl for 5-10 min and washed in distilled water followed by incubation in PBS for 15 minutes. Staining was done in DAPI staining solution for 30 minutes in dark condition and washed in PBS by a modified method (Begum & Alam, 2016). Root tips were squashed and mounted in 50% glycerol. Slides were then prepared and the number of damaged cells and total cells were scored in 6 different fields of view using 40X of the fluorescent microscope (Leica DFC 450C, Germany) for cytogenetic effects. Mitotic index (%) and aberration percentage (%) were calculated using the following formulae and values were expressed as mean±SE from at least three independent experiments:

$$\text{Mitotic Index (\%)} = \frac{\text{Number of dividing cells}}{\text{Total number of cells}} \times 100$$

$$\text{Aberration percentage (\%)} = \frac{\text{Number of aberrated cells}}{\text{Total number of cells}} \times 100$$

In situ *visualization of cell death*

For the assessment of cell death, control and treated bulbs with intact roots were placed in Evans blue staining solution for 15 min, followed by washing of the roots in running tap water for 30 min (Baker & Mock, 1994). Subsequently, 10 root tips measuring equal length (10 mm) from control and the treated groups were excised and soaked in 3 mL of N, N-dimethylformamide for 1 h at room temperature. The absorbance of Evans blue released was measured spectrophotometrically at 600 nm (Elico SL 218, India).

RESULTS

GC/MS analysis

The volatile composition of methanolic extract of *C. viminale* was determined by GC/MS. A total of 26 compounds were detected in the methanolic extracts of *C. viminale* by GC/MS. These compounds belonged to various classes *viz*., terpenoids, aldehydes, fatty acids, phenolics, fatty acid esters *etc*. The compounds identified in the methanolic extract of *C. viminale* by GC-MS analysis are enlisted in Table 1 and gas chromatogram is given as Fig. 1. The major compounds detected were carvone (31.57%), hexadecanoic acid (29.56%) and 9-cis-octadecenoic acid (10.57%). Terpenes were the predominant class of compounds present in the extract; also aldehydes and alcohols in significant quantities. 2-hexyl-2-decenal, pentadecanal, and myristaldehyde were the aldehydes present in the extract. Coniferyl alcohol, 2,4,4-trimethyl-2-penten-1-ol, (E)-2-nonenol and 1-heptanol were the alcohols present in the extract. Nonanoic acid methyl ester, heptadecanoic acid methyl ester, isopropyl pentadecanoate and methyl docosanoate were the fatty acid esters present in the extract. Phenolic compounds like p-vinylguaiacol, 3-tert-butyl-4-methoxyphenol and allylsyringol were detected in negligible amounts. An alkaloid, 6-bromo-5-methoxy-Nb methoxycarbonyltryptamine was also detected in the analysis. The extract contained fatty acids such as myristic acid,

Table 1. Chemical composition of *C. viminale* as analysed by GC/MS.

Sl No.	RT	Compounds	Class	Content (%)
1	6.58	Limonene	Terpene	8.06
2	**9.68**	**Carvone**	**Terpene**	**31.57**
3	10.98	p-vinylguaiacol	Phenol	0.37
4	14.25	3-tert-butyl-4-methoxyphenol	Phenol	0.43
5	15.85	Allylsyringol	Phenol	0.96
6	16.33	Coniferyl alcohol	Alcohol	5.34
7	16.35	2-nitropropane	Alkane	0.12
8	17.24	Myristaldehyde	Aldehyde	1.64
9	17.69	Nitrous acid, butyl ester	Carboxylic acid ester	0.13
10	18.12	Nonanoic acid, methyl ester	Fatty acid ester	0.47
11	18.45	Myristic acid	Fatty acid	0.56
12	**18.55**	**Hexadecanoic acid**	**Fatty acid**	**29.56**
13	18.82	2-methyl 1-butanol nitrite	Organic compound	0.1
14	18.91	Acetic acid, methyl ester	Carboxylic acid ester	0.11
15	18.95	6-bromo-5-methoxy-Nb methoxycarbonyltryptamine	Alkaloid	0.81
16	19.97	Phytol	Diterpene alcohol	0.95
17	**20.28**	**9-cis-octadecenoic acid**	**Fatty acid**	**10.57**
18	22.33	Pentadecanal	Aldehyde	0.37
19	22.63	Isopropyl pentadecanoate	Fatty acid ester	0.9
20	24.11	2,4,4-trimethyl-2-penten-1-ol	Alcohol	0.33
21	24.23	Methyl docosanoate	Fatty acid ester	0.12
22	24.27	(E)-2-nonenol	Alcohol	0.25
23	24.58	4-methyl pentanoic acid	Carboxylic acid	0.66
24	36.20	1,5-diazabicyclo[5.4.0]undec-5-ene	Amide	0.73
25	37.33	2-hexyl-2-decenal	Aldehyde	0.34
26	39.38	1-heptanol	Alcohol	4.55

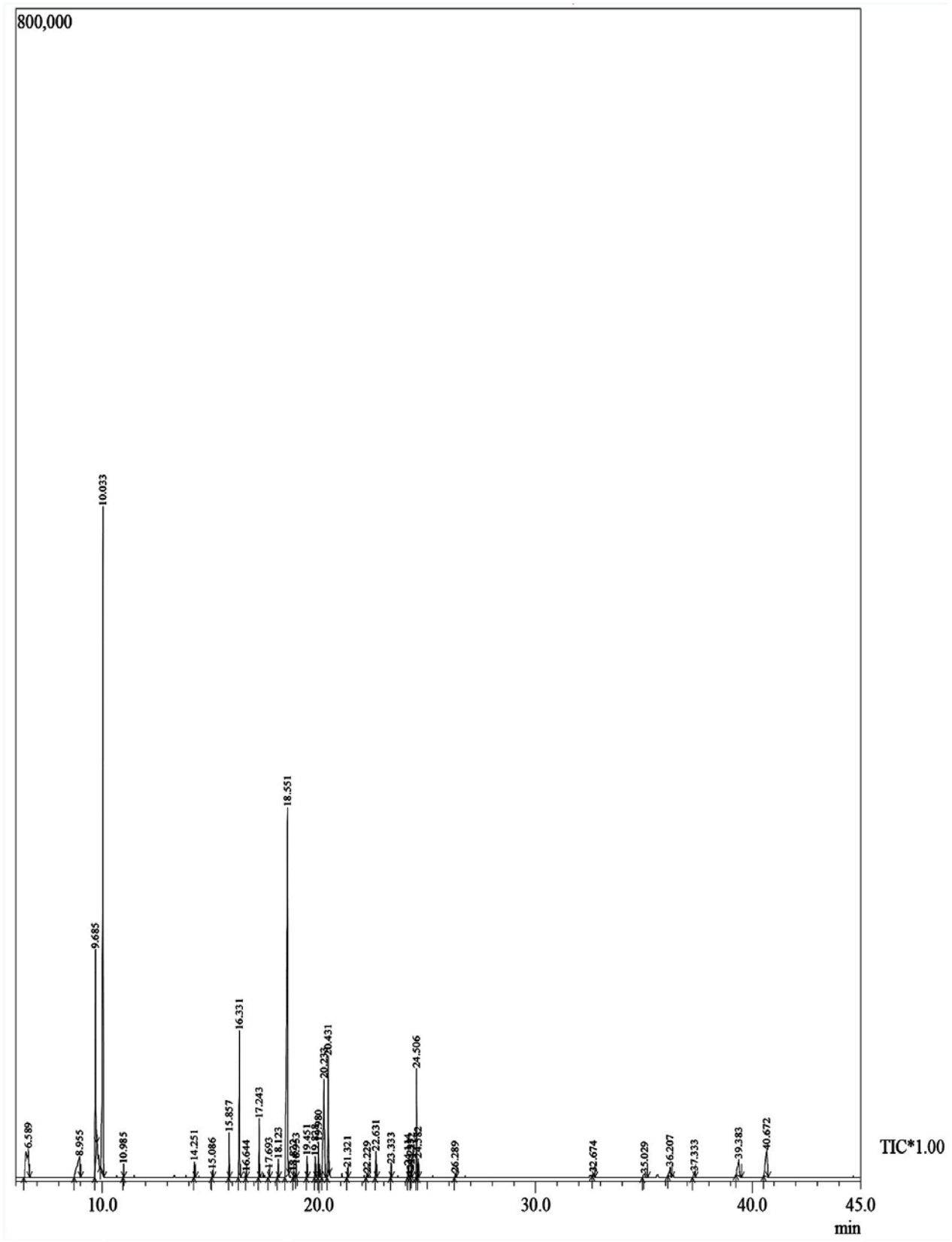


Fig. 1. GC chromatogram of methanolic extract of *C. viminale* [Total ion current (TIC) chromatogram].

9-cis-octadecenoic acid, tridecanoic acid and hexadecanoic acid. Among these, hexadecanoic acid was the predominant one. Negligible quantity of carboxylic acid esters *viz*., nitrous acid butyl ester and acetic acid methyl ester were also present in the extract. Other compounds belonging to amides, alkanes, alkenes, alkynes and heterocyclic organic compounds *etc*. were also detected in trace quantity.

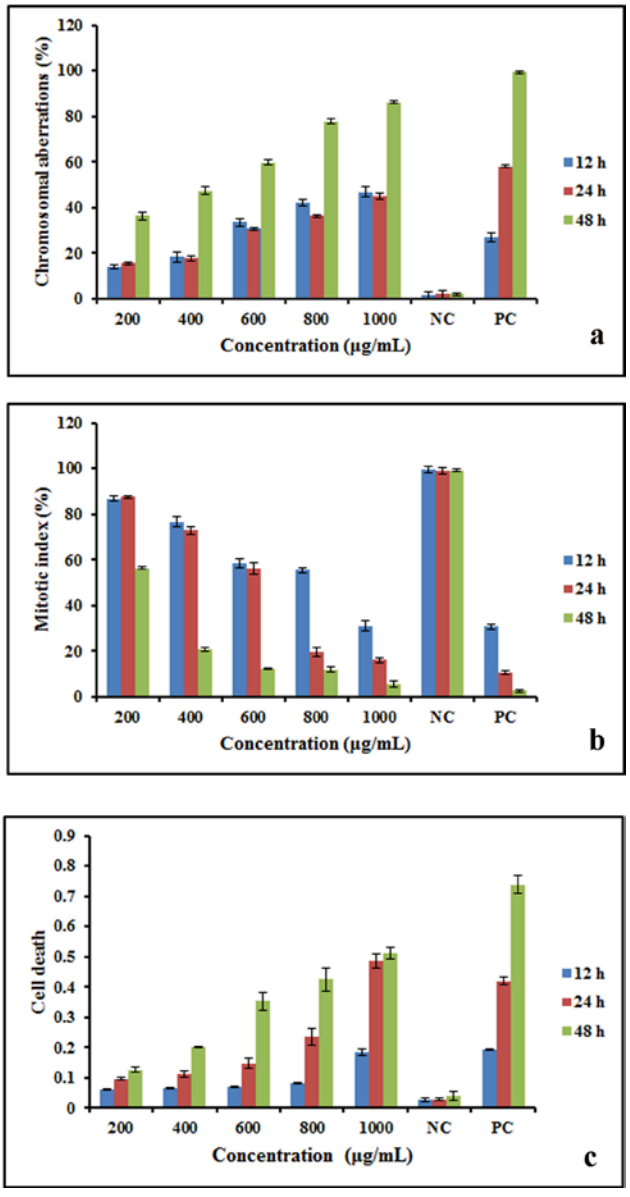


Fig. 2. a: Determination of chromosomal aberrations on *A. cepa* by *C. viminale;* b: Mitotic index; c: Spectrophotometric determination of cell death by Evans blue staining.

Cytotoxic evaluation on A. cepa *root meristem*

Cytotoxic potential of *C. viminale* in terms of mitotic index and chromosomal aberrations were tested on *A. cepa* root tips. The concentrations of methanolic plant extracts of 200, 400, 600, 800 and 1000 µg/mL as well as incubation period of 12 h, 24 h, and 48 h were taken as the experimental conditions. Time-and dose-dependent increase in chromosome aberrations were observed in *A. cepa* as visualized by DAPI staining.

Effect on mitotic index

Reduction in mitotic index is an important factor concerning the cytotoxicity of plant extracts on *A. cepa*. At 12 h period of incubation, the percentage of dividing cells was 86.66 ± 1.07 in the 200 µg/mL concentration of *C. viminale* (Fig. 2b). On increasing concentration, mitotic index is found to be gradually declined with respect to concentration and exposure time. At the highest concentration, 1000 µg/mL of *C. viminale,* mitotic index was observed as 31.11 ± 2.24%. In *C. viminale* extract treatment, mitotic index was found to be even lower than the positive control. The decrease in mitotic index was positively correlated with an increasing concentration of plant extracts. In addition to concentration, the time period is an important factor in genotoxicity and reduction of mitotic index. At the final time period of 48 h, mitotic index (5.47 ± 0.62%) was declined to much lower percentage in all concentrations tested than other two time periods considered. In the case of positive control, mitotic index was found to sharply decreased to 2.55 ± 0.56% at 48 h where was in negative control group, no reduction in mitotic index was observed. The progressive reduction in the number of dividing cells at increasing concentrations of plant extracts suggests that the plant extract has a mitodepressive effect on the cell division of *A. cepa*.

Effect on chromosomal aberrations

Chromosomal aberration percentage is also an endpoint parameter considered for cytotoxicity assays. Time-and dose-dependent increase in chromosome aberrations was observed in *A. cepa* exposed to plant extracts (Fig. 2a). At the lowest concentration 200 µg/mL, chromosome aberrations were 13.98 ± 1.74%. As observed in the case of mitotic index of *A. cepa* root cells treated with plant extract, dose- and time-dependent variation of chromosome aberrations were also observed. During 12 h treatment period and at the high-

est concentration of methanolic extracts of *C. viminale,* chromosome aberrations observed were 46.88 ± 0.68%. Hydrogen peroxide was used as the positive control, which induced serious clastogenic aberrations in *A. cepa* root cells in the form of prominent nuclear lesions. However, the plant extracts at 600, 800 and 1000 µg/mL concentrations induced more aberrations than the positive control. In the case of chromosomal aberrations, it was increased up to 86.24 ± 0.68 % for *C. viminale* at 1000 µg/mL concentration for 48 h.

Wide spectra of chromosomal aberrations were induced by the plant extract, more specifically numerous apoptotic symptoms were found to be prominent. The major chromosomal aberrations observed in the study was lesions, nuclear budding, nuclear peak, nuclear extrusion, nuclear fragmentation, bridged binucleate cell, giant cells, nuclear disintegration, nuclear erosion, hyperchromasia, fragmentation, cytoplasmic vacuolation *etc.* (Fig. 3-4). Nuclear buds were observed as frequent chromosomal aberration observed in higher concentration of plant extract and its various stages of development were also observed (Fig. 5). It is noteworthy to observe the apoptotic symptoms such as apoptotic bodies, nuclear disintegration, micronucleus *etc.* in *A. cepa* cells treated with different concentrations of *C. viminale* plant extract. Most of the damages were multiple aberrations such as bridged binucleate cell, giant cell with cytoplasmic shrinkage, shrunken and twisted cell with nuclear diminution, double budding and lesion, chromosome fragmentation in the hypoploid cell *etc.* which indicated the acute cytotoxic potential of the species of *Cynanchum*. These results suggested the significant cytotoxic potential or more specifically, genotoxic potential of methanolic extracts of *C. viminale* on *A. cepa* meristematic cells mediated by apoptotic signs.

In-situ *visualization of cell death*

Visualization of cell death of *A. cepa* root cells was performed by Evans blue staining and their corresponding estimation of cell death was carried out spectrophotometrically at 12, 24 and 48 h of treatment periods. N, N-dimethylformamide was the solvent used to release Evans blue from root cells and the solvent containing Evans blue was then quantified by noting their absorbance. Spectrophotometric determination of cell death suggested that severe cytotoxicity was observed in the higher concentration of plant extracts at 48 h of the incubation period.

At 12 h of incubation of *A. cepa* root cells with methanolic extracts of *C. viminale*, absorbance was found to be gradually increasing with respect to the concentration. Furthermore, cell death was highest in positive control and minimum for negative control. Dose and time served as an important factor concerning the cell death of *A. cepa* by methanolic extracts of *C. viminale*. Dosage and exposure time was found to be directly proportional to cell death. Incubation of *A. cepa* root cells with methanolic extracts of *C. viminale* for 24 h resulted in the cell death of maximum absorbance 0.48 ± 0.02 (Fig. 2c). Finally, incubation of *A. cepa* with methanolic extracts of *C. viminale* for 48 h caused a profound cell death. The absorbance read was 0.51 ± 0.01, which corresponds to the cell death. Negative control exerted negligible cell death and positive control treated *A. cepa* showed extremely severe cell death in terms of 0.73 ± 0.03 absorbance.

DISCUSSION

Methanol has a higher dielectric constant than ethanol; which enables to extract more polar compounds in comparison with ethanol. As a safety concern, methanol content is completely removed by evaporating the extract and thus the further studies were carried out with various concentration of extracts prepared in water. The GC/MS analysis revealed the presence of 26 constituents in the methanolic extract of *C. viminale* (Table 1). The peak with a maximum area of intensity of 31.57% corresponds to carvone followed by hexadecanoic acid (29.56%) and 9-cis-octadecenoic acid (10.57%). Carvone is a monoterpene found as an important constituent of essential oil of spearmint, clove, syzygium *etc.* (Kokkini et al. 1995; Chaieb et al. 2007) and found to have insecticidal and genotoxic activity. Apart from these, limonene is another monoterpene that occupied 8.06% of the total area. The cytotoxic activity of limonene was evaluated in amelanotic melanoma C32, renal cell adenocarcinoma ACHN, hormone-dependent prostate carcinoma LNCaP, and MCF-7 breast cancer cell lines by the sulfo rhodamine B assay (Loizzo et al. 2007). p-vinyl guaiacol is a phenolic component present in 0.37% peak area. Also, the compound is a major constituent of almost all essential oils from plants (*Bituminaria*, *Ferula*, *Torreya etc.*) as reported before. 3-tert-butyl-4-methoxyphenol is a phenolic constituent and a potential antioxidant compound which was recognized from the essential oil of *Dictamnus dasycarpus* which showed significant antimicrobial activity and cytotoxicity towards ACHN, MCF-7, ZR-75-30, MDA-MB-435S, Hep-G2 and Bel-7402 cell lines (Lei et al. 2008).

Coniferyl alcohol is present as 5.34% of the peak area of the total area of intensity. It is synthesized *via*

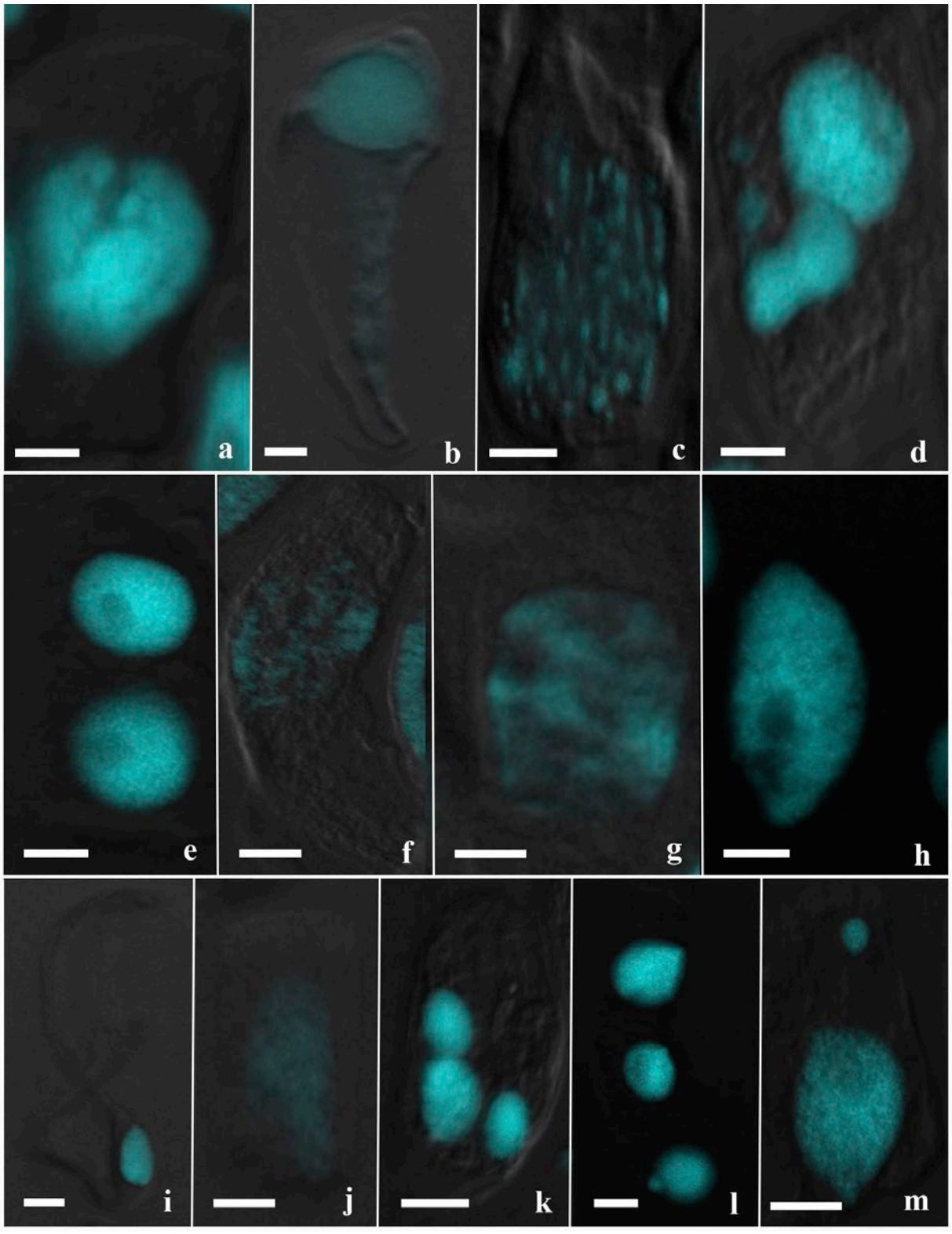


Fig. 3. Chromosomal aberrations induced by extract of *C. viminale* on *A. cepa*. a: apoptotic breakage of nucleus at interphase; b: cytoplasmic vacuolation; c: apoptotic fragmentation of nucleus; d: binucleate cell showing micronuclei; e: binucleate cell with lesions; f: nuclear disintegration; g: apoptotic nuclear disintegration; h: nuclear peak; i: shrunken and twisted cell with nuclear diminution; j: nuclear disintegration; k: trinucleate cell; l: trinucleate cell showing different stages of nuclear budding; m: nuclear budding and micronucleus; Bar: 10 μm.

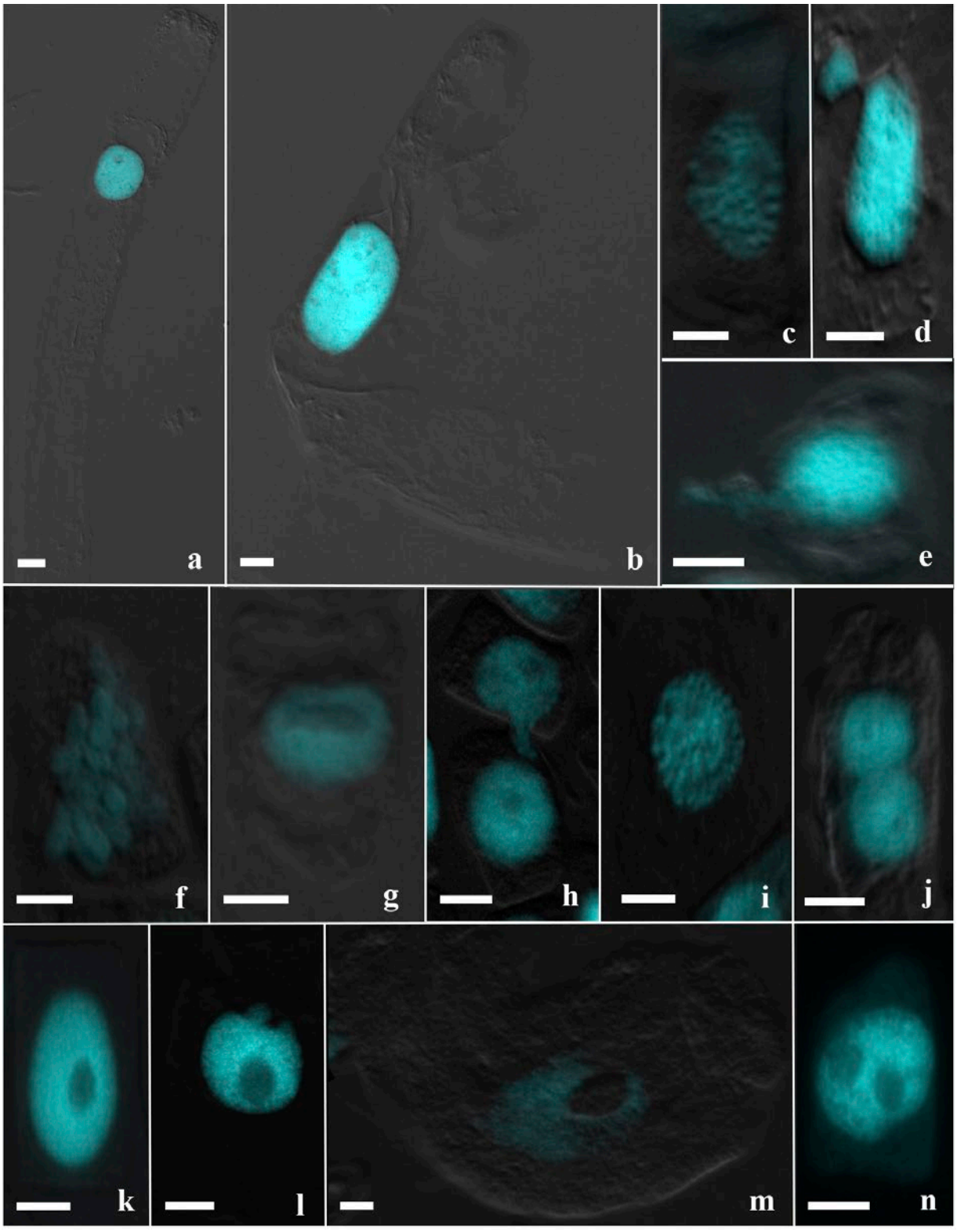


Fig. 4. Chromosomal aberrations induced by extract of *C. viminale* on *A. cepa*. a: giant cell; b: giant cell with cytoplasmic shrinkage; c: formation of apoptotic bodies in the nucleus; d: bridged binucleate cell; e: nuclear extrusion; f: apoptotic body formation; g: nuclear and cytoplasmic lesions; h: cytomictic transfer of nuclear material; i: nuclear disintegration; j: binucleate cell; k: nuclear lesion; l: double budding and lesion; m: giant cell showing nuclear disintegration and lesion; n: double nuclear lesions; Bar: 10 μm.

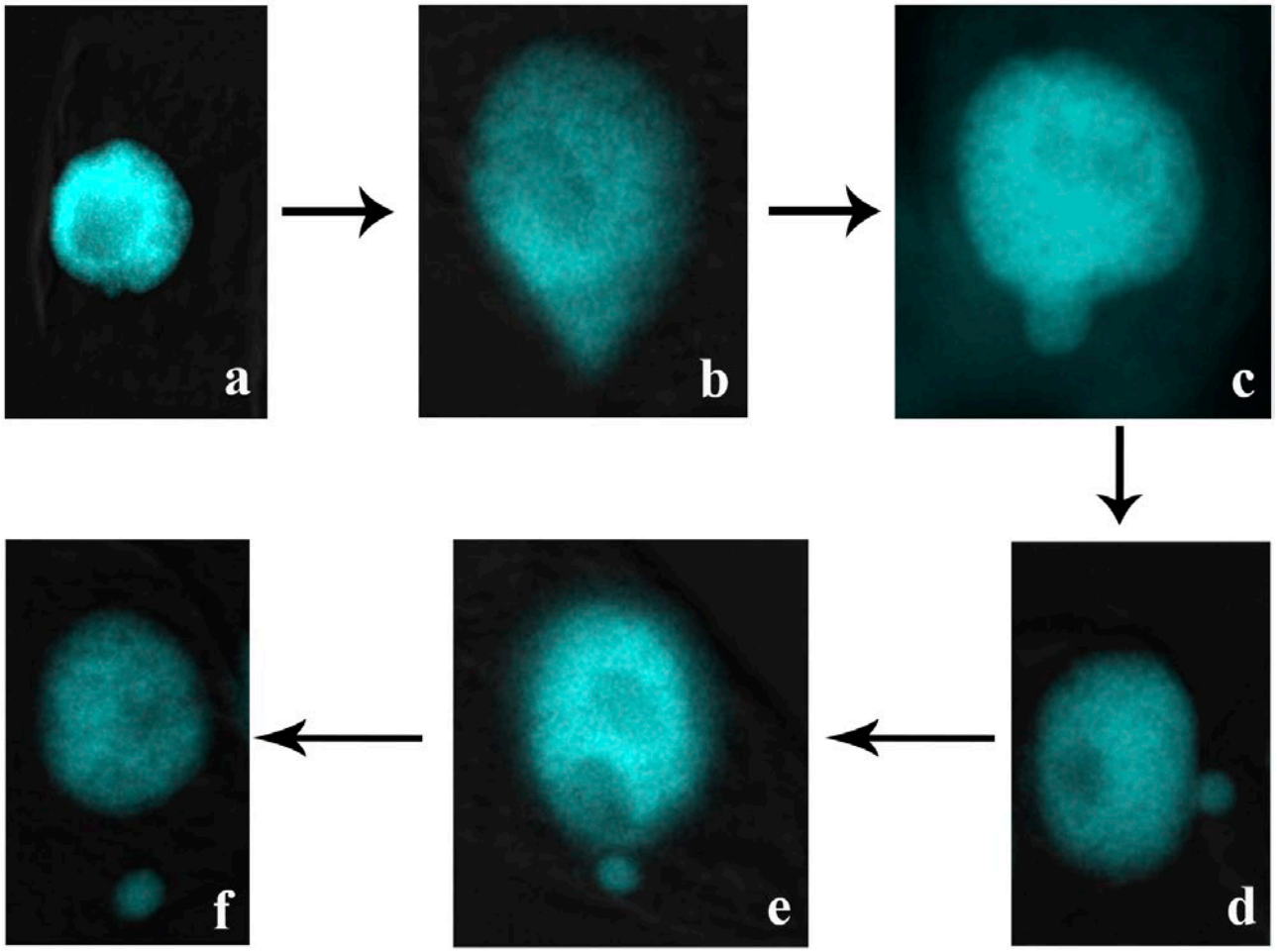


Fig. 5. Various stages of nuclear budding induced by extract of *C. viminale*. a: initiation of bud; b: growth of bud; c: protruding as fully formed bud; d: bud with a basal notch; e: detachment of bud from the nucleus; f: micronucleus.

the phenylpropanoid biochemical pathway and it is an intermediate in the biosynthesis of eugenol, stilbenoids, and coumarin (Kadir et al. 2015). Myristic acid is another fatty acid component present in 0.56% in the whole content of plant extract. Earlier the antioxidant and larvicidal activity against malaria and filariasis vectors were studied using the bioactive fraction of myristic acid from *Ammannia baccifera* aerial extract (Suman et al. 2013). Phytol is another compound detected (0.95%) and it is a diterpene alcohol found ubiquitously in many plant species. It was well documented that phytol is having cancer preventive properties irrespective of their concentration in the plant (Hema et al. 2011).

In this study, *C. viminale* contained a bioactive fatty acid hexadecanoic acid which was found to be predominant (29.56%). Hexadecanoic acid, a palmitic acid type compound was detected in extracts of various plants and have shown to possess hemolytic, antioxidant, anticancer, nematicide, 5-alpha reductase inhibition properties *etc*. (Jananie et al. 2011; Kalaivani et al. 2012). A systematic study with respect to the fatty acid composition of *Sarcostemma viminale* has been carried out earlier, where hexadecanoic acid and octadecanoic acid were the major components (Girme et al. 2014). 9-cis-octadecenoic acid, another fatty acid present as 10.57% of the total content of volatile compounds present in *C. viminale*. The derivatives or their esters have the potential to act against cancer or prevent cancer at the very initial stage itself (Farina & Chodahry, 2011).

The present study evaluates cytotoxic efficacy of *C. viminale* mediated by acute apoptotic symptoms in *A. cepa* root cells. Several researchers had demonstrated the efficacy of *A. cepa* bioassay for validating the cytotoxic potential of plants. The present observations showed a sharp decline in the mitotic index of *A. cepa* root cells as a result of treatment with different concentrations of the

plant extract, which is a clear indication of the mitotic depressive effect of the crude plant extracts. The positive control used for the study was H_2O_2, a serious clastogen which directly interacts with genetic material and result in prominent nuclear lesions in *A. cepa* which suggest that it interfere with cell cycle mechanism at the initial stage itself; so cells couldn't be passed onto the next stages of cell cycle.

The aberrations induced by plant extract had the potential to affect all phases of cell cycle. Henceforth, these results suggest that the tested concentrations of *C. viminale* extract is inhibitory, turbagenic and mitodepressive on cell division of *A. cepa*, which is in agreement with Akintonwa et al. (2009). The genotoxic effect of *C. viminale* was evidenced by a remarkable lowering of mitotic division in vegetative cells of *A. cepa*. In the experiments, mitotic activity showed a tendency to decrease to 5.47 ± 0.62% respectively for *C. viminale* at the highest concentration (1000 µg/mL) of plant extract at 48 h treatment. This reduction in the mitotic activity could be attributed to inhibition of DNA synthesis or blockage of the cell cycle in G2 phase, thus preventing the cells from entering into mitosis (Sudhakar et al. 2001).

Many serious chromosomal aberrations were observed as a result of treatment with various concentrations of plant extract. Of these, 90% of the damages were contributed to the genotoxic aberrations. Treatment of *A. cepa* with *C. viminale* extract resulted in various apoptotic symptoms like nuclear buds, micronuclei, nuclear fragmentation, nuclear blebbing *etc.* Most of the damages were nucleotoxic, whereas other aberrations were caused by the disturbance on the formation of spindle fibers during cell division. Nuclear buds are one of the prominent aberrations observed in the bioassay experiment. Four models have been proposed for the generation of nuclear buds. Nuclear buds are formed in the S-phase, representing the expulsion of excess genetic material derived from the polyploidization process, which may subsequently lead to micronucleation (Fernandes et al. 2007; Lindberg et al. 2007)micronucleus-like bodies attached to the nucleus by a thin nucleoplasmic connection, have been proposed to be generated similarly to micronuclei during nuclear division or in S-phase as a stage in the extrusion of extra DNA, possibly giving rise to micronuclei. To better understand these phenomena, we have characterized the contents of 894 nuclear buds and 1392 micronuclei in normal and folate-deprived 9-day cultures of human lymphocytes using fluorescence in situ hybridization with pancentromeric and pantelomeric DNA probes. Such information has not earlier been available for human primary cells. Surprisingly, there appears to be no previous data on the occurrence of telomeres in micronuclei (or buds, whose chromatin replication has failed. Nuclear bud formation from broken anaphase bridges (Gisselsson and Pettersson 2000) would appear to be an clear explanation, assuming that the typically stalked structure of a bud results from the collapse of the bridge when it is resolved.

The mechanisms responsible for micronucleus have not been yet fully understood. It may have originated during anaphase from lagging acentric chromosomes or chromatid fragments caused by misrepair of DNA breaks or unrepaired DNA breaks (Fenech et al. 2011; Bonciu et al. 2018). Nuclear blebs were also observed in *A. cepa* cells, consisting of nuclear material, with bud-shaped excrescences on the main nucleus, protruded from the nucleus, but without an obvious constriction or bridge between the protruding nuclear material and nucleus (Wang et al. 2014). Nuclear lesions and erosions are a type of nuclear disintegration, observed frequently in *A. cepa* cells as a result of treatment with *C. viminale* extract. These may suggest the direct action of phytochemical components on DNA synthesis and it is a cytological evidence for the inhibitory action on DNA biosynthesis and nuclear poisoning (Saghirzadeh et al. 2008; Ngozi, 2011). Nuclear erosion, which may result from the disintegration of chromatid proteins, represents irreversible toxicity (Karaismailoglu et al. 2013). Nuclear extrusion or sometimes nucleolar extrusion was another type of clastogenic event frequently observed in *A. cepa* cells. It is known that the nuclear pore complex (NPC) was the most important channel for nuclear material transport. The phenomenon that the nucleolar material was extruded from the nucleus into the cytoplasm could be explained by the fact that the proteins were affected after plant extract treatment, causing the NPC to lose selectivity (Qin et al. 2010). The fragmentation of nuclei may indicate cell death process and this may ultimately result in aneuploidy and then to cell death. This pattern of nuclear degeneration of nucleus were also observed in programmed cell death in the nucellus of *Tillandsia* presenting various signals of degeneration like deformed shape, chromatin condensation, plasmalemma detachment etc. (Brighigna et al. 2006). Binucleate and trinucleate cells were the frequent aberrations observed in the study, due to the inhibition of cytokinesis in any of the control points of the cell cycle (Özkara et al. 2015). Moreover, shrunken root cells, nuclear blebs, marked nuclear chromatin condensation, fragmentation *etc.* clearly indicate the possibilities to tend towards apoptosis. These clastogenic, as well as apoptotic signs of aberrations, provide a clue that the plant *C. viminale* can be effectively utilized for anticancer studies.

In addition, it is interesting to highlight the high frequency of multiple chromosomal aberrations [bridged

binucleate cell, giant cell with cytoplasmic shrinkage, chromosome fragmentation in a hypoploid cell, giant cell showing nuclear disintegration and lesion, double nuclear lesions *etc.*] in cells of *A. cepa* rather than single aberration by treatment with *C. viminale* extracts. The above results point to the phytochemicals present in the extracts which might have disrupted the cell cycle mechanism since various cytotoxic compounds such as carvone, limonene *etc.* were detected in GC/MS analysis. They might have possibly interfered with the normal cell cycle process and led to cell death. The present results thus support the notion that the cytotoxic effect of plant extracts is due to the synergistic action of a broad array of phytochemicals, the total activity of which may result in health benefits. Moreover, multiple aberrations of chromosomes might have attributed by the multiple compound-multiple target mechanism of interaction between phytochemical constituents of *C. viminale* and *A. cepa* cells.

Cytotoxic efficacy of *C. viminale* was then confirmed by estimating the cell death of *A. cepa* root cells. Evans blue staining method works on the basis of its penetration to non-viable cells (Panda et al. 2011). Evans blue staining of treated and control roots of *A. cepa* points is considered as an indirect evidence of cell death by visualising the intensity of Evans blue taken up by roots, suggesting the loss of viability of cells. The intensity of dye absorbed by root cells was directly proportional to the cell death; this could be seen within few minutes after the treatment, in corroboration with the result reported earlier (Achary et al. 2008). Cell death can be positively correlated with an increase in the concentration of plant extract and increase in duration of treatment.

CONCLUSION

The cytotoxic effects were found to increase proportionately with the concentration of plant extract. The chromosomal aberrations observed in this study are evidently caused by the chemical constituents in the extract since no aberration was observed in the negative control. The above obtained cytotoxic results may account for the severe cell death and this observation provides a plausible basis for its further use in anti-proliferative studies on *in vitro* cancer cell lines. However, the mechanism of action remains to be investigated in plant test system and further studies are necessary to clarify the fact.

ACKNOWLEDGEMENT

The first author gratefully acknowledges the Department of Science and Technology, Government of India (C/2003/1FD/2014-15) for the financial assistance in the form of INSPIRE fellowship. Thanks are also due to Advanced Instrumentation Research Facility, Jawaharlal Nehru University for the GC-MS facility.

REFERENCES

Adams RP 2001. Identification of Essential Oils by Capillary Gas Chromatography/Mass Spectroscopy, Allured, Carol Stream, IL, USA.

Akintonwa A, Awodele O, Afolayan G, Coker HAB 2009. Mutagenic screening of some commonly used medicinal plants in Nigeria. J Ethnopharmacol 125: 461–470.

Begum K, Alam S 2016. Karyomorphological analysis with differential staining of nine *Cicer arietinum* L. varieties. Bangl J Bot 45(2), 327–334.

Baker C, Mock N 1994. An improved method for monitoring cell death in cell suspension and leaf disc assays using Evans blue. Plant Cell, Tissue and Organ Culture, 39(1), 7–12.

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

Chaieb K, Hajlaoui H, Zmantar T, Kahla-Nakbi A Ben, Rouabhia M, Mahdouani K, Bakhrouf A 2007. The chemical composition and biological activity of clove essential oil,Eugenia caryophyllata (Syzigium aromaticum L. Myrtaceae): a short review. Phyther Res 21: 501–506. doi: 10.1002/ptr.2124

Cilla A, Attanzio A, Barberá R, Tesoriere L, Livrea MA 2015. Anti-proliferative effect of main dietary phytosterols and β-cryptoxanthin alone or combined in human colon cancer Caco-2 cells through cytosolic Ca+ 2– and oxidative stress-induced apoptosis. J Functl Foods 12: 282–293.

Farina Asghar S, Choudahry MI 2011. Gas chromatography-mass spectrometry (GC-MS) analysis of petroleum ether extract (oil) and bio-assays of crude extract of Iris germanica. Int J Genet Mol Biol 3: 95–100.

Fenech M, Kirsch-Volders M, Natarajan AT, Surralles J, Crott JW, Parry J, Norppa H, Eastmond DA, Tucker JD, Thomas P 2011. Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. Mutagenesis 26: 125–132.

Fernandes TCC, Mazzeo DEC, Marin-Morales MA 2007. Mechanism of micronuclei formation in polyploidi-

zated cells of Allium cepa exposed to trifluralin herbicide. Pestic Biochem Physiol 88: 252–259.

Girme AS, Bhalke RD, Nirmal SA, Chavan MJ 2014. Chromatographic and chemical analysis of Sarcostemma viminale R. Br. Orient Pharm Exp Med 14: 279–284. doi: 10.1007/s13596-014-0157-3

Gisselsson D, Pettersson L 2000. Chromosomal breakage-fusion-bridge events cause genetic intratumor heterogeneity. Proceedings of the National Academy of Sciences, 97(10), 5357-5362

Hema. R, Kumaravel. S, Alagusundaram. K 2011. GC/MS Determination of Bioactive Components of *Murraya koenigii*. J Am Sci 7: 2009–2012.

Jananie R, Priya V, Vijayalakshmi K 2011. Determination of bioactive components of Cynodon dactylon by GC-MS analysis. New York Sci J 4: 16–20.

Kadir R, Awang K, Khamaruddin Z, Soit Z 2015. Chemical compositions and termiticidal activities of the heartwood from *Calophyllum inophyllum* L. An Acad Bras Cienc 87: 743–751.

Kalaivani CS, Sathish SS, Janakiraman N, Johnson M 2012. GC-MS studies on Andrographis paniculata (Burm.f.) Wall. ex Nees - A medicinally important plant. Int J Med Arom Plants 2: 69–74.

Karaismailoglu, M. C., Inceer, H., & Ayaz, S. H. 2013. Effects of Quizalofop-p-ethyl herbicide on the somatic chromosomes of *Helianthus annus* (sunflower). Ekoloji, *89*, 49–56.

Khanna N, Sharma S 2013. *Allium cepa* root chromosomal aberration assay: A review. Ind J Pharm Biol Res 1(3), 105–119.

Kokkini S, Karousou R, Lanaras T 1995. Essential oils of spearmint (Carvone-rich) plants from the island of Crete (Greece). Biochem Syst Ecol 23: 425–430.

Lei J, Yu J, Yu H, Liao Z 2008. Composition, cytotoxicity and antimicrobial activity of essential oil from Dictamnus dasycarpus. Food Chem 107: 1205–1209.

Leme DM, Marin-Morales MA 2009. *Allium cepa* test in environmental monitoring: A review on its application. Mutat Res – Reviews in Mutat Res 682(1), 71–81.

Lindberg HK, Wang X, Järventaus H, Falck GCM, Norppa H, Fenech M 2007. Origin of nuclear buds and micronuclei in normal and folate-deprived human lymphocytes. Mutat Res - Fundam Mol Mech Mutagen 617: 33–45.

Loizzo MR, Tundis R, Menichini F, Saab AM, Statti GA, Menichini F 2007. Cytotoxic activity of essential oils from Labiatae and Lauraceae families against in vitro human tumor models. Anticancer Res 27: 3293–3299.

Meve U, Liede-Schumann S 2012. Taxonomic dissolution of *Sarcostemma* (Apocynaceae: Asclepiadoideae). Kew Bullet 67(4), 751–758.

Achary MMV, Jena S, Panda KK, Panda BB 2008. Aluminium induced oxidative stress and DNA damage in root cells of Allium cepa L. Ecotoxicol Environ Saf 70: 300–310.

Ngozi E 2011. Mutagenicity testing of pharmaceutical effluents on *Allium cepa* root tip meristems. J Toxicol Envtl Health Sci 3(2), 44–51.

Özkara A, Akyıl D, Eren Y, Erdoğmuş SF 2015. Potential cytotoxic effect of Anilofos by using Allium cepa assay. Cytotechnol 67: 783–791.

Panda K, Achary V, Krishnaveni R, Padhi B 2011. In vitro biosynthesis and genotoxicity bioassay of silver nanoparticles using plants. Toxicol. In vitro. 25(5): 1097-105.

Qin R, Jiao Y, Zhang S, Jiang W, Liu D 2010. Effects of aluminum on nucleoli in root tip cells and selected physiological and biochemical characters in *Allium cepa* var. *agrogarum* L. BMC Plant Biol 10: 225.

Saghirzadeh M, Gharaati MR, Mohammadi S, Ghiassi-Nejad M 2008. Evaluation of DNA damage in the root cells of Allium cepa seeds growing in soil of high background radiation areas of Ramsar - Iran. J Environ Radioact 99: 1698–1702.

Sudhakar R, Gowda KNN, Venu G 2001. Mitotic abnormalities induced by silk dyeing industry effluents in the cells of Allium cepa. Cytologia (Tokyo) 66: 235–239.

Suman TY, Elumalai D, Kaleena PK, Rajasree RSR 2013. GC-MS analysis of bioactive components and synthesis of silver nanoparticle using *Ammannia baccifera* aerial extract and its larvicidal activity against malaria and filariasis vectors. Ind Crops Prod 47: 239–245.

Wang QL, Zhang LT, Zou JH, Liu DH, Yue JY 2014. Effects of cadmium on root growth, cell division and micronuclei formation in root tip cells of *Allium cepa* var. *agrogarum* L. ΦYTON 83: 291–298.

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: S. Chatterjee, S. Ray (2019) Clastogenic and cytotoxic effects of aerial parts' aqueous extract of *Synedrella nodiflora* (L.) Gaertn. on Wistar rat bone marrow cells. *Caryologia* 72(3): 87-95. doi: 10.13128/caryologia-770

Published: December 13, 2019

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

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

Clastogenic and cytotoxic effects of aerial parts' aqueous extract of *Synedrella nodiflora* (L.) Gaertn. on Wistar rat bone marrow cells

Saumabha Chatterjee[1,2], Sanjib Ray[2,*]

[1] *Department of Zoology, Durgapur Govt. College, Durgapur-713214*
[2] *Molecular Biology and Genetics Unit, Department of Zoology, The University of Burdwan, Golapbag, Burdwan-713104, West Bengal, India*
*Corresponding author: ray.sanjibray@gmail.com

Abstract. *Synedrella nodiflora* is a traditionally used medicinal plant. The aim of the present study was to analyze the clastogenic and cytotoxic effects (CCEs) of aerial parts' aqueous extract of *S. nodiflora* (AAESN) on Wistar rat bone marrow cells (WRBMCs). The CCEs of AAESN were analyzed with light and fluorescence microscopes respectively. The data indicate a dose-dependent (100-500 mg/kg body weight [bw]) increase in the aberrant cell frequencies, chromosomal structural aberrations and a significant ($p<0.001$) increase in apoptosis in the AAESN treated WRBMCs. The chromosomal aberration per 100 cells (apoptosis %) were calculated as 2.01±0.241 (5.02±1.72), 4.76±0.05 (46.73±2.34), 5.37±0.32 (66.92±2.92) and 6.58±0.14 (76.79±0.73) respectively for the AAESN doses of 0, 100, 300 and 500 mg/kg bw. In conclusion, the AAESN may contain phytochemicals with clastogenic and cytotoxic efficacy on WRBMCs, indicating, having the anticancer as well as carcinogenic potentials. Therefore, it demands further an elaborate study to explore the active principle(s) and a proper care should be taken while it is prescribed in traditional medicine.

Keywords. Apoptosis, Clastogenic, Cytotoxic, Synedrella nodiflora, Genotoxic, Anticancer, Secondary metabolites.

1. INTRODUCTION

Cancer is an important worldwide health problem and the antiproliferative pharmacological activities of plant-derived secondary metabolites appear to explain the anticancer effects (Figueroa-Hernandez *et al.* 2005). A variety of bioactive components were isolated from the different medicinal herbs (Cragg *et al.* 1996). Alkaloids, fixed oils and fats, polyphenols, flavonoids, saponins, glycosides, terpenoids *etc*., having medicinal value, were extracted from a wide variety of plant species (Tamilarashi *et al.* 2000). Many plant-based active compounds act as antitumor and apoptotic cell death inducer in tumors (Sato *et al.* 1994). Acetogenins like uvaribonin, 22-epicalmistrin, and chalcone showed significant antiproliferative activity against a panel of cancer cell lines (Pettit *et al.* 2008). Generally, the cell cycle components are the

prime targets of most of the efficient anticancer agents (Li *et al.*, 2002). The discovery of competent anticancer drugs like vincristine and vinblastine were isolated from *Catharanthus roseous;* Paclitaxel (Taxol®) extracted from *Taxus brevifolia* represent trustworthy proof that plants are potential sources of novel anticancer chemotherapeutic drugs (Cragg *et al.*, 1996). The antiproliferative, clastogenic, and cytotoxic pharmacological activities of plant-derived secondary metabolites appear to elucidate the chemo-preventive or anticancer effects. Therefore, searching for the phytochemicals having the clastogenic and cytotoxic effects (CCEs) are of renewed interest in drug discovery for cancer treatment.

The CCEs of a medicine provide a glimpse into its mechanism of action. A clastogen can cause chromosomal structural alterations like chromatid break, deletion, sister chromatid exchanges, sister chromatid union, dicentric chromosome, acentric fragments, micronuclei *etc.* that subsequently may lead to the various cytotoxic effects including cell killing, apoptosis, and necrosis. Moreover, the medicinal plants having these effects, one can also assume their toxicity risk factor for its indiscriminate use in the traditional therapeutic purpose (Thybaud *et al.* 2007). Cancer chemotherapeutic drugs are generally cytogenotoxic, hence subjected to non-target destruction but non-cancerous cells can revive better than the cancerous one (Choudhury *et al.* 2000; Palo *et al.* 2009). Since these clastogenic chemotherapeutic drugs might enhance the chance of secondary cancer development, dose optimization, target specification, and combination chemotherapy with the other antioxidants are highly recommended (Pandit and Choudhury, 2011). Many anti-cancer agents cause DNA damage at a very high level leading to the cell cycle checkpoint activation and programmed cell death (Helleday *et al.* 2008). Administration of the many plant-derived anticancer agents, including paclitaxel, can affect spindle stability leading to abnormal mitosis and chromosomal aberration (Dumontet and Jordan, 2010).

Synedrella nodiflora (L.) Gaertn. (Family: Asteraceae) is an ephemeral flowering weed. It is indigenous to tropical America and also distributed in India, Malaysia, Bangladesh, China, Japan, and other Indopacific countries (Wiart 2006). In India, *S. nodiflora* leaves are traditionally applied for the remedy of rheumatism. In Ghana, oral application of warm aqueous juice of this plant results in the remedy of epilepsy. In Malaysia, it is used externally as a medicine for the treatment of inflammation, headache, and earache. The leaves are also used for the treatment of hiccup, stomachache, and threatened abortion cases (Rathi and Gopalkrishnan 2005; Rahmatullah *et al.* 2010; Bhogaonkar *et al.* 2011). The toxicological (Olukunle and Abatan 2008; Dutta *et al.* 2012), insecticidal (Rathi and Gopalkrishnan, 2005), larvicidal (Ghayal *et al.* 2010), antibacterial, antioxidant (Wijaya *et al.* 2011), antidiarrhoeal, hypoglycaemic (Zahan *et al.*, 2012), anti-inflammatory properties (Haque *et al.* 2012) of this plant have been reported. Our previous study revealed the antiproliferative activity of the aerial parts' aqueous extract of *S. nodiflora* (AAESN) on root apical meristem cells and Wistar rat bone marrow cells (WRBMCs) as well as the presence of different phytochemicals like alkaloids, flavonoids, terpenoids, tannins, phlobatannins, and saponins in the AAESN (Ray *et al.* 2013b). However, the CCEs of AAESN on the mammalian system has not been well-studied. Thus, the present study is focused on the assessment of the clastogenic and cytotoxic effects of the aerial parts' aqueous extract of *S. nodiflora* on WRBMCs *in vivo* condition. Nabeel *et al.* (2008) described the cytogenetic effect of the aqueous extract of *Arum maculatum* on the Bone Marrow Cells of the Swiss male mice. Some of the parameters recorded by the scientists were also taken into consideration in this study. The novel aspects of this study are that it explored the CCEs of AAESN, a source of future anticancer chemotherapeutic drugs, and raised the question against its indiscriminate use in traditional medicine.

2. MATERIALS AND METHODS

2.1. Chemicals

Colchicine, glacial acetic acid, and methanol were obtained from BDH Chemicals Ltd., UK. EDTA was procured from Gibco, Grand Island, N.Y, USA. Ethidium bromide and acridine orange were purchased from Sigma, St. Louis, M.O., USA and S.D. Fine-Chem. Ltd., Mumbai, India respectively. Other chemicals used in this work were of analytical grade from reputed manufacturers.

2.2. Plant products collection, storage, and extract preparation

Plant aerial parts collection, storage, and extract preparation procedures were described in detail in our earlier report (Ray *et al.* 2013b) and briefly the fresh aerial parts' of *S. nodiflora* were collected from Golapbag campus of The University of Burdwan, taxonomically authenticated by Prof. Ambarish Mukherjee, and the voucher specimen (No.BUGBSC013) is maintained in the Department (Figure 1). The dried and pulverized plant product was boiled in double-distilled water

Fig. 1. Showing the aerial parts (leaf, stem, and flower) of *Synedrella nodiflora* (L.) Gaertn.

(1:10, W/V) in a water bath for 30 min. The extract was allowed to cool to room temperature, filtered by Whatman filter paper No. 1 (Sigma-Aldrich, Inc., St. Louis, MO, USA), and then refrigerated at -20°C for further use. For the measurement of extract value (17.64%w/w) and extract concentration (11.3 mg/mL), 10 mL of extract was kept for evaporation to complete dehydration in a hot air oven at 60 °C.

2.3. Experimental animals

Male Wistar-albino rats (age 4-6 weeks; weight 40–60 g) were purchased from local vendors and maintained in the Departmental animal house (in community cages) at room temperature (25±2°C), controlled illumination (12 h light and 12 h dark cycle), and with standard rat diet and water. The rules of the "Institutional Animal Care and Use Committee" were strictly followed throughout the whole experiment and the required total 24 rats were euthanized with prior approval from the Dissection Monitoring Committee (DMC) of The University of Burdwan (No: R-S/N-1/646, Dated 30-03-2016; Under Ref. No. BU-DMC/2016/01/05(a) Dated 13.07.2016).

2.4. Treatment and clastogenicity analysis

The AAESN (100, 300 and 500 mg/kg body weight [bw]) was injected into the peritoneal cavity of the male Wistar rats (Ray et al. 2013b). Control rat groups were injected an equal volume of double distilled water. At each data point, six rats were used. After 12 h of AAESN injection, colchicine (10 mg/kg bw), a standard metaphase arresting agent, was injected into the peritoneal cavity of the rats irrespective of control and treatment groups for 3 h (Ray et al 2013b). Then the animals were euthanized by cervical dislocation just before the femur bones were dissected out (Ray *et al.* 2013b) and the WRBMCs were fixed in aceto-methanol (1:3) after the required hypotonic (0.56% KCl) treatment. Control group was considered for nullifying the sole toxic effect of colchicine. The detailed procedure of metaphase plate preparation and Giemsa staining procedure were described earlier (Ray et al. 2013b). Briefly, the femur bones were dissected out, the bone marrow cells were collected in 15 mL centrifuge tubes by flushing with pre-warmed (37°C) 2.5 mL of 0.56% aqueous KCl solution with 5 mL hypodermic syringe, the cells were maintained in a hypotonic solution for 30 min at 37°C in a water bath and then the cells were fixed with aceto-methanol (methanol 3 parts and acetic acid 1 part). Metaphase plate preparation was done through flame-drying technique and 2% Giemsa (staining duration: 35 min) was used for staining. The Giemsa stained slides were mounted with a coverslip in synthetic medium and the different types of chromosomal abnormalities like chromatid break, terminal deletion, fragmented chromosome, centric fusion, centromeric association, ring chromosome, chromatid gap, chromosomal association, end to end association *etc.* were scored (Kumpawat *et al.* 2003).

2.5. Fluorescence microscopic cytotoxicity analysis

The acridine orange-ethidium bromide (AO-EB) double staining procedure (Bustillo *et al.* 2009) was used to determine cytotoxic effects of AAESN in terms of early and late phases of apoptosis with the fluorescence microscope by observing changes in nuclear morphology and apoptotic blebbing. It is the established fact that acridine orange can infiltrate in both the live and dead cells while ethidium bromide enters only in dead cells. The AO-EB staining strategy causes color differentiation with blue-filter excitation; the living cells (stained only with acridine orange) give green fluorescence, the early apoptotic cells (permitting limited penetration of ethidium bromide) show green to yellowish nuclei with perinuclear chromatin condensation, the late apoptotic cells show a dark red color with fragmented or condensed chromatin, and the necrotic cells give red color with large nucleus having no condensed chromatin.

Here, the AAESN treatment and WRBMCs collection procedures were same as described for clastogenicity analysis, except, the harvested cells were washed in 1X PBS instead of hypotonic KCl solution. After cen-

trifugation at 1000 rpm for 5 minutes and discarding the supernatant, the precipitates were stained with acridine orange-ethidium bromide mix (conc. 100 µg/mL) in 1:1 ratio for 5 minutes in 2 mL Eppendorf tubes. Then, cells were washed thrice with 1X PBS repeating the centrifugation steps. The cells were resuspended in 100 µl 1X PBS and then 20 µL cell suspensions were taken on grease free slide, the percentages of dead cells (apoptotic vs necrotic) and live cells were scored under Leica fluorescence microscope.

2.5. Scoring and Statistical analysis

All the results were expressed as Mean±SEM. Aberrant cell percentage were analyzed through one way ANOVA (d.f.=11) followed by Tukey-Kramer tests. The differences between the untreated and treated groups for cellular viability and chromosomal abnormalities were analyzed with the 2x2 contingency χ^2-test and were considered statistically significant at $p<0.05$, $p<0.01$ and $p<0.001$.

3. RESULTS

3.1. Clastogenic effects of AAESN

Metaphase chromosomal abnormalities were scored in all the AAESN treated groups of rats and were compared with the untreated controls. The various clastogenic effects were observed in the AAESN treated rats. The number of cell abnormality percentage was calculated as 4.76±0.03, 5.37±0.32, and 6.58±0.14 after AAESN treatment respectively with 100, 300, and 500 mg/kg bw as compared with the control (2.01±0.24 %) (Table 1). Among the different clastogenic effects scored, chromatid break, terminal deletion, centric fusion, centromeric association, ring chromosome, chromosomal association, and an end to end association followed a dose-dependent increase in frequencies. Here, the chromosomal association (0.98±0.01) percentage was found to be the highest frequency of chromosomal abnormality followed by centric fusion (0.89±0.03), fragmented chromosome (0.88±0.01), end to end association (0.86±0.02), centromeric association (0.83±0.01), chromatid break (0.54±0.03), ring chromosome (0.51±0.06), and terminal deletion (0.45±0.02) after the treatment with 500 mg/kg bw of AAESN (Figure 2 and Table S1).

Table 1. Pooled data showing the AAESN induced aberrant cell percentage of WRBMCs.

AAESN treatment Dose (mg/kg bw)	TM	Ab.Cell(%)= (TAC/TMC)x100	
		Range	Mean±SEM (% increase)
00	100	1.72-2.48	$2.01\pm0.24^{\#\$}$
100	96	4.69-4.84	$4.76\pm0.05^{*\#\$}$ (136.8)
300	94	4.81-5.93	$5.37\pm0.32^{*\#\$}$ (167.2)
500	109	6.42-6.86	$6.58\pm0.14^{*\#\sharp}$ (227.4)

*Significant at $p < 0.05$ as compared to the control, # at $p < 0.05$ as compared to the 100 mg/kg AAESN treatment, ♯ at $p < 0.05$ as compared to the 300 mg/kg AAESN treatment, $ at $p < 0.05$ as compared to the 500 mg/kg AAESN treatment by one way ANOVA (d.f.=11) followed by Tukey-Kramer Procedure. bw; body weight, TM; Total no. of metaphases counted for studying chromosomal abnormality, Ab.Cell %; aberrant cell per percentage; TAC; Total number of aberration count, TMC; Total metaphase count.

3.2. Fluorescence microscopic analysis for cytotoxicity

Apoptotic cells were examined under a fluorescence microscope after acridine orange and ethidium bromide (AO-EB) combined staining of WRBMCs. A dose-dependent increase in the apoptotic cells (%) was observed in AAESN treated samples. As compared with the untreated cells, a significantly ($p<0.001$) increased percentages of early and late apoptotic cells, and necrotic cells were observed in AAESN treated WRBMCs. The dose-dependent response was more prevalent in apoptotic cell death than that of necrosis. The maximum percentages (85.40±1.71) of viable cells were counted in the

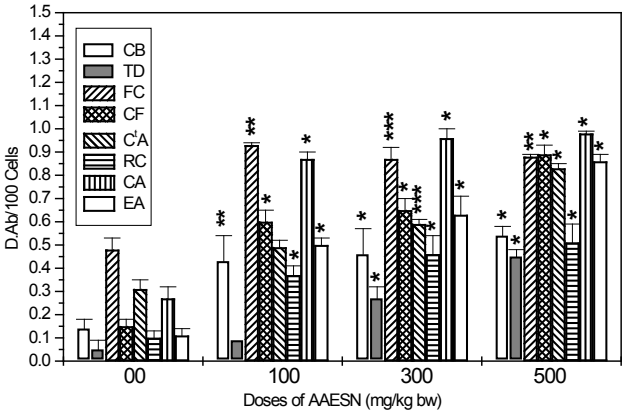


Fig. 2. Influence of AAESN on WRBMCs in terms of different chromosomal abnormalities. *Significant at p<0.05, **at p<0.01 and ***at p<0.001 as compared to the control by 2x2 contingency χ2-test (d.f.=1). The data were represented as Mean±SEM. D.Ab./Cell: Different abnormalities per cell scored; CB: Chromatid break; TD: Terminal deletion; FC: fragmented chromosome; CF: Centric fusion; CtA: Centromeric association; RC: Ring chromosome; CA: Chromosomal association; EA: End to end Association.

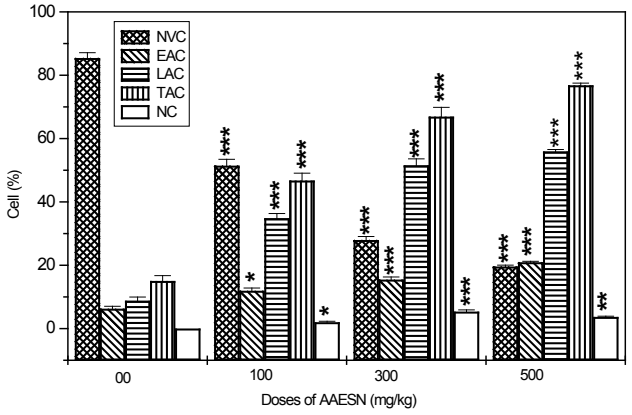


Fig. 3. Shows AAESN induced cytotoxicity observed on WRBMCs under a fluorescent microscope. NVC: normal viable cells; EAC: early apoptotic cells; LAC: late apoptotic cells, TAC: total apoptotic cells. NC: necrotic cells. Experiments were done in three sets and data were represented as Mean±SEM. *Significant at $p<0.05$, **at $p<0.01$ and ***at $p<0.001$ as compared to the control by 2x2 contingency χ^2-test (d.f.=1).

untreated samples and the maximum percentages of early apoptotic (20.92±0.34), late apoptotic (55.87±0.69) and the total apoptotic cell frequency (76.79±0.73) were calculated in AAESN (500 mg/kg bw) treated samples. The maximum percentage (5.34±0.57) of necrotic cells were scored at a dose of 300 mg/kg bw of AAESN for 500 mg/kg of AAESN treatment (Figure 3 and Table S2).

4. DISCUSSION

The leaves of *Synedrella nodiflora* are traditionally used for the remedy of rheumatism, epilepsy, inflammation, headache, stomachache, and earache (Rathi and Gopalkrishnan 2005; Rahmatullah *et al.* 2010; Bhogaonkar *et al.* 2011). In the present study, we have tested *in vivo* CCEs of AAESN on the WRBMCs. The rapidly dividing bone marrow cells are the ideal model to demonstrate antiproliferative activity of herbal extracts (Ray *et al.*, 2013 a, b). The WRBMCs are also considered as a basic model for clastogenicity testing and many authors have used it to study the side-effects of anti-cancer/anti-inflammatory medicines (Pearse *et al.* 2009; Pandit and Choudhury 2011; Haque *et al.* 2012; Ray *et al.* 2013a; Ray *et al.* 2013b; Gomaa 2018). Generally, the bone-marrow mito-depressive drugs show anticancer activity (Prasanthi, 2016). The previous studies indicated the mutagenic activity of pyrethroids on murine bone marrow cells, human peripheral blood lymphocytes, and in aquatic animals (Barrueco *et al.* 1992; Oraby 1997). The general non-steroidal anti-inflammatory drugs (NSAIDs) may also inhibit the proliferation of bone marrow cells without any intervention of hormonal activities in murine models (Chang *et al.*, 2007). The study of the bone marrow suppression is designated as a common toxicity assessment of cytotoxic agents and this toxicity assay had been included in the preclinical study of the four-stage trial system (Heidelberg and Fox, 1990).

Our earlier study indicated a dose-dependent decrease in metaphase frequency (Ray *et al.* 2013b) and in the present study a dose-dependent increase in chromosomal abnormalities (both total and differential counts) and apoptotic cells percentage in WRBMCs. Several anticancer agents put forth their influence through cell cycle events (Salmon *et al.*, 1984). The antiproliferative activities of the several herbal extracts are related to their ability to obstruct DNA synthesis (Akinboro and Bakare 2007; Mercykutty and Stephen 1980). *Toona sinensis* leaf aqueous extract has been reported to have an anti-proliferative influence on human lung cancer cells (Laosinwattana *et al.* 2007, 2009).

In the present study, clastogenicity of AAESN on the WRBMCs revealed that several types of chromosomal abnormalities including chromosomal fragmentation, chromatid break, ring chromosome formation, centromeric association *etc.* were induced by AAESN. Kumpawat *et al.* (2003) showed that raw betel-nut extract introduced clastogenicity on mouse bone marrow cells and human peripheral blood lymphocytes. There are similar kinds of study reports where they explored the genotoxic activity of plant extracts on WRBMCs and human peripheral blood lymphocytes (Pandit and Choudhury 2011; Sakamoto-Hojo *et.al.* 2017).

We previously reported the cytogenotoxic alteration of onion apical meristem cells that were exerted by the aerial parts' aqueous extract of *Ampelocissus latifolia* (Chaudhuri and Ray 2014). Nefic (2008) described the effect of ascorbic acid on human peripheral blood lymphocytes, where vitamin-C at a higher concentration (1,000µg/mL) could induce mitotic arrest and chromosomal abnormalities. The similar kinds of dose-optimization studies were performed using the methanolic extracts of *Artemisia annua* and *Pyracantha coccinea* on *Allium cep*a root apical meristem cells (Karaismailoglu MC 2014, 2017). Pandit and Choudhury (2011) narrated the clastogenic effect of a chemotherapeutic drug on mouse bone marrow cells. Gewirtz (1999) revealed cytogenotoxic effect of anthracyclin antibiotics due to suppression of Topoisomerase-II and thus hindering Topoisomerase-II mediated DNA cleavage and re-ligation. Moreover, it also triggers ROS generation (Dorosho, 1983).

Many anti-cancer agents cause DNA damage at a high level leading to checkpoint activation and programmed cell death (Helleday *et al.* 2008). Administration of anticancer agents like paclitaxel can affect spindle stability leading to abnormal mitosis and chromosomal aberration (Dumontet and Jordan, 2010). One noticeable thing is that colchicine, a mitostatic drug, was also used here to arrest the cells at metaphase but it was equally administered to both control and treatment groups. Here, the treatment group showed a significantly higher level of clastogenic alterations of chromosomes in comparison to the control group in a dose-dependent manner. So, apart from colchicine, there must be extra clastogenic effects of AAESN. We previously reported cell cycle retardation effect as well as the increased prophase-metaphase frequency on *Allium cepa* root apical meristem cells treated by the same extract. Recently, Bonciu *et al.* (2018) used *Allium cepa* root apical meristem cells as a genotoxicity test system. The mito-retarding effect was also noticed in case of WRBMCs in that study in a dose-dependent manner, apart from the influence of colchicine in both control and treatment groups (Ray *et al.*, 2013b). Hence, the strong probability of interaction of the phytochemical(s) present in AAESN with mitotic spindle could not be ignored. However, a detailed mechanism of clastogenic action of AAESN is subjected to further detailed study.

The AO-EB combined staining assay data indicate a dose-dependent increase in the apoptotic cell frequency to a much greater extent than that of necrotic cells. Treatment with AAESN caused the characteristic changes related to apoptotic morphologies in WRBMCs indicating that *S. nodiflora* is an extensive source of natural bioactive substances with apoptotic cell death-inducing activity on WRBMCs. Another important observation was that the necrotic cells' increased frequency did not follow a dose-response relationship and the data, in turn, suggest an apoptotic potential of AAESN. The maximum percentage (5.34±0.57) of necrotic cells were scored at a dose of 300 mg/kg bw of AAESN, indicating, unlike apoptosis, the necrotic cell frequencies did not follow a dose-dependent response pattern.

There are similar reports showing the apoptotic cell death-inducing effects of some of the anticancer agents like isodeoxyelephantopin (Farha *et al.*, 2013) and farnesiferol c (Hasanzadeh *et al.*, 2017). Previously, we also described the cytotoxic effect of aerial parts' aqueous extract of *A. latifolia* on apical meristem cells using AO-EB staining method (Chaudhuri and Ray 2014). Chu *et al.* (2014) showed the antiproliferative and cytotoxic effect of Camptothecin-20(s)-*O*-(2-pyrazolyl-1) acetic ester (CPT6) on breast tumor MCF-7 cells by increased sub G_1 cell population and apoptosis induction among the treatment groups. Farha *et al.* (2013) reported isodeoxyelephantopin (IDOE) mediated apoptosis on nasopharyngeal carcinoma (KB) cells by obtaining more apoptotic morphologies through AO-EB combined staining assay in IDOE treated KB cells. Our results are in agreement with those of Ichikawa who reported the apoptosis-inducing effects of isodeoxyelephantopin in various cells (Ichikawa *et al.*, 2006).

Our previous study revealed the presence of alkaloids, tannins, terpenoids, flavonoids, phlobatannins, and saponins as active ingredients in this extract (Ray *et al.* 2013b). These active ingredients might have interacted with the cell cycle machinery and/or with DNA thus inducing cell cycle delay, clastogenicity, and apoptosis. The AAESN-induced abnormal mitosis in WRBMCs might lead to the mitotic catastrophe through apoptosis and necrosis.

Mitotic catastrophe is an intrinsic mechanism that senses mitotic failure / abnormal mitosis and responds by driving a cell to an irreparable antiproliferative fate of death or senescence (Margaret 2015; Vakifahmetoglu *et al.* 2008). Here, the mitotic catastrophe induced by AAESN was more prone to result in apoptosis rather than necrosis which is more desired outcome in cancer chemotherapy. Our results related to toxicity of this plant are in agreement with the histopathological toxicity and brine shrimp lethality of *S. nodiflora* (Olukunle *et al.* 2008; Dutta *et al.* 2012).

5. CONCLUSION

The phytochemicals present in aerial parts' aqueous extract of *Synedrella nodifolia* could induce cytotoxicity, clastogenicity and mitotic catastrophe in WRBMCs and thus indicate its potential use in cancer chemotherapy in the near future. There is ample scope to explore the active principle(s) of AAESN and the detailed molecular mechanism of the CCEs. Moreover, awareness should be raised among the concerned tribals regarding its probable side-effects due to indiscriminate use and overdose.

FUNDING

Financial support was received from the UGC (JRF), India and infrastructural supports received from DST-PURSE, FIST, UGC-DRS to the Department of Zoology, The University of Burdwan was used to execute the work.

REFERENCES

Akinboro A, Bakare AA. 2007. Cytotoxic and genotoxic effects of aqueous extracts of five medicinal plants on *Allium cepa*. Linn. J. Ethnopharmacol. 112(3): 470-475.

Barrueco C, Herrera A, Cabolla C, Pena EDL. 1992. Cytogenetic effects of permethrin in cultured human lymphocytes. Mutagenesis. 7(6): 433-437.

Bhogaonkar PY, Dagawal MJ, Ghorpade DS. 2011. Pharmacognostic studies and antimicrobial activity of *Synedrella nodiflora* (L.) Gaertn. Biosci. Discov. 2 (3): 317-321.

Bonciu *et al.* 2018. An evaluation for the standardization of the *Allium cepa* test as cytotoxicity and genotoxicity assay. Caryologia, 71(3): 191-209

Bustillo S, Lucero H, Leiva LC, Acosta OKJEB, Gorodner JO. 2009. Cytotoxicity and morphological analysis of cell death induced by *Bothrops* venoms from the Northeast of Argentina. J. Venom. Anim. Toxins Incl. Trop. Dis. 15(1): 28-42.

Chang JK, Li CJ, Wu SC, Yeh CH, Chen CH, Fu YC, Wang GJ, Ho ML. 2007. Effects of anti-inflammatory drugs on proliferation, cytotoxicity and osteogenesis in bone marrow mesenchymal stem cells. Biochem. Pharm. 74(9): 1371-1382.

Chaudhuri A, Ray S. 2014. Evaluation of phytotoxic and cytogenotoxic potentials of leaf aqueous extract of *Ampelocissus latifolia* (Roxb.) planch. in relation to its total polyphenol content. Int. J. Pharm. Bio Sci. 5(4): 225–235.

Choudhury RC, Ghosh SK, Palo AK, 2000. Cytogenetic toxicity of methotrexate in mouse bone marrow. Env. Toxicol. and Pharm. 8: 191-196.

Chu C, Jialin X, Dongping C, Xingnuo L, Shengqiang T, Jizong Y, Qingyong L. 2014. Anti-proliferative and apoptosis-inducing effects of Camptothecin-20(s)-*O*-(2-pyrazolyl-1) acetic ester in human breast tumor MCF-7 Cells. Molecules. 19: 4941-4955.

Cragg GM, Simon JE, Jato JG, Snader KM. 1996. Drug discovery and development at the National Cancer Institute: potential for new pharmaceutical crops, En: Janick J. ed., Progress in New Crops, (Arlington, VA: ASHS Press.) p.554-560.

Doroshow JH, 1983. Anthracycline antibiotic stimulated superoxide, hydrogen peroxide, and hydroxyl radical production by NADH dehydrogenase. Cancer Res. 43: 4543-4551.

Dumontet C, Jordan MA. 2010. Microtubule-binding agents: a dynamic field of cancer therapeutics. Nat Rev. Drug Discov. 9(10): 790-803.

Dutta M, Nath AK, Uddin MZ, Hossain MA, Morshed MM, Kawsar MH. 2012. *In vitro* antioxidant, total phenolic content and brine shrimp lethality studies of *Synedrella nodiflora*. *Int.* J. Pharm. Sci. Res. 3(5): 1528-1531.

Farha AK, Geetha BS, Mangalam S, Nair DSR, Latha PG, Remani P. 2013. Apoptosis mediated cytotoxicity induced by isodeoxyelephantopin on nasopharyngeal carcinoma cell. Asian J. Pharm.Clin. Res. 6(2): 51-56.

Figueroa-Hernandez JL, Gonzalez GS, Ascencio VJ, Figueroa-Espiti JL, Saavedra GF. 2005. Plant products with anti-cancer properties employed in the treatment of bowel cancer: literature review 1985 and 2004. Proc. West Pharmacol. Soc. 48: 77-83.

Gewirtz DA. 1999. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. Biochem. Pharmacol. 57: 727- 741.

Ghayal N, Padhye A, Kondiram D. 2010. Larvicidal activity of invasive weeds *Cassia uniflora* and *Synedrella nodiflora*. Int. J. Pharm. Bio Sci. 1(3): 245-249.

Gomaa S. 2018. Adverse Effects Induced by Diclofenac, Ibuprofen, and Paracetamol Toxicity on Immunological and Biochemical Parameters in Swiss Albino Mice. The J. Basic Appl. Zool. 79(1): 1-9.

Haque A, Zahan R, Nahar L, Mosaddik A, Haque E. 2012. Anti-inflammatory and insecticidal activities of *Synedrella nodiflora*. Mol. Clin. Pharm. 2(2): 60-67.

Hasanzadeh D, Mahdavi M, Dehghan G, Charoudeh HN. 2017. Farnesiferol C induces cell cycle arrest and apoptosis mediated by oxidative stress in MCF-7 cell line. Toxicol. Rep. 4: 420–426.

Heidelberg DS, Fox BW. 1990. Cytostatic Anticancer Drug Development: Preclinical Studies and Early Clinical Trials. Oncol. Res. and Treatment- Congress Report. 13(1): 57-60.

Helleday T, Petermann E, Lundin C, Hodgson B, Sharma RA. 2008. DNA repair pathways as targets for cancer therapy. Nat. Rev. Cancer, 8: 193–204.

Ichikawa H, Nair MS, Takada Y, Alan SDB, Kumar S, Oommen VO, Agarwal BB. 2006. Isodeoxyelephantopin, a novel sesquiterpene lactone, potentiates apoptosis, inhibits invasion, abolishes osteoclastogenesis through suppression of Nuclear Factor k B activation and Nuclear Factor k B regulated gene expression. Clin. Cancer. Res. 12(19): 5910-5918.

Karaismailoglu MC 2014. Investigation of the Cytotoxic and Genotoxic Effects of *Artemisia annua* Methanol Extract with the *Allium* Test. Ekoloji, 23 (91): 64-74.

Karaismailoglu MC 2017. Investigation of the antimitotic and antimutagenic effects of methanolic extracts of *Pyracantha coccinea*. Turkish J. of Life Science 2(1): 110-116.

Kumpawat K, Deb S, Ray S, Chatterjee A. 2003. Genotoxic effect of raw betel-nut extract in relation to endog-

enous glutathione levels and its mechanism of action in mammalian cells. Mutat. Res. 538: 1–12.

Laosinwattana C. Phuwiwat W, Charoenying P. 2007. Assessment of allelopathic potential of Vetivergrass (*Vetiveria* sp.) ecotypes. Allelopath. J. 19 (2): 469-477.

Laosinwattana C. Poonpaiboonpipat T, Teererak M, Phuwiwat W. Mongkolaussavaratana T, Charoenying P. 2009. Allelopathic potential of Chinese rice flower (*Aglaia odorata* Lour.) as organic herbicide. Allelopath. J. 24(1): 45-54.

Li Y, Shan F, Wu JM, Wu GS, Ding J, Xiao D, Yang WY, Atassi G, Leonce S, Caignard DH, Renard P. 2002. Novel antitumor artemisinin derivatives targeting G1 phase of the cell cycle. Bioorg. Med. Chem. Lett. 11: 5-8.

Margaret MMG. 2015. Targeting the mitotic catastrophe signaling pathway in cancer. Int. Sch. Res. Not. Hindawi. 2015: 1-13.

Mercykutty VC, Stephen J. 1980. Adriamycin-induced genetic toxicity as demonstrated by the *Allium* test. Cytologia. 45(4): 769-777.

Nabeel M, Abderrahman S, Papini A. 2008. Cytogenetic Effect of *Arum maculatum* Extract on the Bone Marrow Cells of Mice. Caryologia, 61(4): 383-387.

Nefic H. 2008. The Genotoxicity of Vitamin C *in vitro*. BOSNIAN J. Basic Med. Sci. 8(2): 141-146.

Olukunle, JO, Abatan MO. 2008. The toxicological effects of aqueous leaf extract of *Synedrella nodiflora* in rats. Asset Ser. B. 7(1): 81-89.

Oraby HA. 1997. Micronuclei formation in bone marrow cells of rats treated with meothrin (synthetic pyrethroid). J. Appl.toxicol. 17(6): 353-356.

Palo AK, Sahoo D, Choudhury RC, 2009. Cytosine arabinoside-induced cytogenotoxicity in bone marrow and spermatogonial cells of mice and its potential transmission through the male germline. Mutat. Res. 673: 29-36.

Pandit RS, Choudhury RC. 2011. Clastogenic effects of the anticancer drug Epirubicin on mouse bone marrow cells. Biol. and Med. 3(5): 43-49.

Pearse G, Pietersma A, Cunliffe J, Foster JR, Turton J, Derbyshire N, and Randall KJ. 2009. Time-course study of the immunotoxic effects of the anticancer drug Chlorambucil in the rat. Toxicol. Pathol. 37: 887-901.

Pettit GR, Mukku VJ, Craqq G, Herald DL, Knight JC, Herald CL, Chapuis JC. 2008. Antineoplastic agents.558. *Ampelocissus* sp. Cancer cell growth inhibitory constituents. J. Nat. Prod. 71(1): 130-133.

Prasanthi D. 2016. Anticancer drugs used in treatment of curing cancer, Research & Reviews: J. Pharmacol. Toxicol. Stud. 4(3): 1-7.

Rahmatullah MK, Rahman AABT, Hossan MM, Khatun MS, Khatun Z, Jahan MAAR. 2010. Ethnomedicinal practices among a minority group of Christians residing in Mirzapur Village of Dinajpur District, Bangladesh. Adv. Nat. Appl. Sci. 4(1): 45-51.

Rathi, MJ, Gopalkrishnan S. 2005. Insecticidal activity of aerial parts of *Synedrella nodiflora* (L.) Gartn (Compositae) on *Sapodeptera latura* (FAB). J. Cent. Eur. Agric. 6: 323-328.

Ray S, Chatterjee S, Chakrabarti CS. 2013b. Antiproliferative activity of allelochemicals present in aqueous extract of *Synedrella nodiflora* L. Gaertn. in apical meristems and Wistar rat bone marrow cells. IOSR J. Pharm. 3(2): 1-10.

Ray S, Kundu LM, Goswami S, Roy GC, Chatterjee S, Dutta S, Chaudhuri A, Chakrabarti CS. 2013a. Metaphase arrest and delay in cell cycle kinetics of root apical meristems and mouse bone marrow cells treated with leaf aqueous extracts of *Clerodendrum viscosum* Vent. Cell Prolif. 46: 109–117.

Sakamoto-Hojo ET, Catarina I, Takahashi S, Ferrari I, Motidome M. 1988. Clastogenic effect of the plant alkaloid ellipticine on bone marrow cells of Wistar rats and on human peripheral blood lymphocytes. Mutat. Res. 199: 11-19.

Salmon ED, Mickseel M, Hays T. 1984. Rapid rate of tubulin dissociation from microtubule in the mitotic spindle *in vivo* measured by blocking polymerization with colchicine. J. Cell Biol. 99(3): 1066-1075.

Sato KM, Mochizuki I, Saiki YC, Yoo K, Samukawa I, Azuma I. 1994. Inhibition of tumor angiogenesis and metastasis by a saponin of *Panax ginseng*, ginsenoside-Rb2. Biol. Pharm. Bull. 17: 635-639.

Tamilarashi CT, Subasini U, Kavimani S, Jaykar B. 2000. Phytochemical and pharmacological evaluation of *Ampelocissus latifolia*. Anc. sci. life. 20(1- 2): 14-18.

Thybaud V, Aardema M, Clements J, Dearfield K, Galloway S, Hayashi M, Jacobson-Kram D, Kirkland D, MacGregor JT, Marzin D, Ohyamaj W, Schuler M, Suzuki H, Zeiger E. 2007. Strategy for genotoxicity testing: Hazard identification and risk assessment in relation to *in vitro* testing. Mutat. Res. 627: 41-58.

Tuhba TT, Gowder S. 2014. Pharmacology and Therapeutics. Intech. The Instrumentation, Systems. The Shard. Chapter 9, Anticancer Drug- Friend or Foe; p. 225-269.

Vakifahmetoglu H, Olsson M, Zhivotovsky B. 2008. Death through a tragedy: Mitotic Catastrophe. Cell Death and Differentiation 15: 1153–1162.

Wiart C. 2006. Medicinal plants of the Asia-pacific: drugs for the future? World Scientific, Kuala Lumpur, Malaysia; p. 635.

Wijaya S. Nee T K, Jin K, Hon TLK, San LH, Wiart C. 2011. Antibacterial and antioxidant activities of *Synedrella nodiflora* (L.) Gaertn. (Asteraceae). J. Complement. Integr. Med. 8(1): 1-13.

Zahan R, Nahar L,. Haque A, Mosaddik A, Faza, A, Alam Z, and Haque ME. 2012. Antidiarrhoeal and hypoglycemic effects of *Synedrella nodiflora*. Phytopharm. 2(2): 257-264.

Table S1. Supplementary pooled data of Fig.2 showing the AAESN induced different categories of aberrant cell percentage of WRBMCs.

Doses (mg/kg bw) of AAESN	TM	D.Ab/Cell (%)							
		CB	TD	FC	CF	C^{t}A	RC	CA	EA
00	100	0.14±0.04	0.05±0.04	0.48±0.05	0.15±0.03	0.31±0.04	0.1±0.03	0.27±0.05	0.11±0.03
100	96	0.43±0.11^b	0.09±0.00	0.93±0.01^b	0.60±0.05^c	0.49±0.03	0.37±0.04^c	0.87±0.03^c	0.5±0.03^c
300	94	0.46±0.11^c	0.27±0.05^c	0.87±0.05^a	0.65±0.05^c	0.59±0.02^a	0.46±0.08^c	0.96±0.04^c	0.63±0.08^c
500	109	0.54±0.04^c	0.45±0.03^c	0.88±0.01^b	0.89±0.04^c	0.83±0.02^c	0.51±0.08^c	0.98±0.01^c	0.86±0.03^c

^{a}Significant at p<0.001, ^{b}at p<0.01 and ^{c}at p<0.05 as compared to the control by 2x2 contingency χ2-test (d.f.=1). TM; Total no. of metaphases counted for studying chromosomal abnormality, D.Ab./Cell: Different abnormalities per cell scored; CB: Chromatid break; TD: Terminal deletion; FC: fragmented chromosome; CF: Centric fusion; C^{t}A: Centromeric association; RC: Ring chromosome; CA: Chromosomal association; EA: End to end Association.

Table S2. Supplementary pooled data of Fig. 3 showing AAESN induced apoptosis and necrosis in WRBMCs *in vivo*.

Doses (mg/kg bw) of AAESN	TC	NVC		EAC		LAC		TAC		NC	
		TC	Mean±SEM	TC	Mean±SEM	TC	Mean±SEM	TC	Mean±SEM	TC	Mean±SEM
00	1728	1476	85.40±1.71	102	6.26±0.78	150	8.75±1.23	252	15.02±1.72	0	-
100	2015	1035	51.44±2.00^a	240	11.93±0.89^c	700	34.80±1.54^a	940	46.73±2.34^a	40	2.00±0.33^c
300	1677	468	27.92±1.16^a	258	15.43±0.86^a	861	51.49±2.12^a	1119	66.92±2.92^a	90	5.34±0.57^a
500	2066	404	19.57±0.45^a	432	20.92±0.34^a	1154	55.87±0.69^a	1586	76.79±0.73^a	76	3.68±0.24^b

Conc.: Concentration; TC: total cells; NVC: normal viable cells; EAC: early apoptotic cells; LAC: late apoptotic cells, NC: necrotic cells, TAC: total apoptotic cells ^{a}Significant at *p<0.001* as compared to the control by 2x2 contingency χ^2-test (d.f.=1). Experiments were done in triplicate and data were represented as Mean±SEM.

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

Citation: D. Şendoğan, B Gündoğan Yağbasan, M.V. Nabozhenko, B. Keskin, N. Alpagut Keskin (2019) Cytogenetics of *Accanthopus velikensis* (Piller et Mitterpacher, 1783) (Tenebrionidae: Helopini). *Caryologia* 72(3): 97-103. doi: 10.13128/caryologia-771

Published: December 13, 2019

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

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

Cytogenetics of *Accanthopus velikensis* (Piller et Mitterpacher, 1783) (Tenebrionidae: Helopini)

Dirim Şendoğan[1], Beril Gündoğan[1], Maxim V. Nabozhenko[2,3], Bekir Keskin[1], Nurşen Alpagut Keskin[1,*]

[1] *Faculty of Science, Department of Zoology, Section of Biology, Ege University, İzmir, Turkey*
[2] *Precaspian Institute of Biological Resources of the Daghestan Federal Research Centre of the Russian Academy of Sciences, Makhachkala, Russia*
[3] *Dagestan State University, Makhachkala, Russia*
*Corresponding author: nursen.alpagut@ege.edu.tr

Abstract. The karyotype and cytogenetic features of darkling beetle *Accanthopus velikensis* were analysed using conventional and differential staining. The diploid number was determined as 2n = 20 and the presence of Xy_p sex determination system was observed with DAPI and silver staining as well as conventional staining. Although a single nucleolar material was observed in prophase I nuclei, multiple argyrophilic signals in diakinesis-metaphase I plates makes it difficult to determine the exact NOR location. Both conventionally and differentially stained plates showed that heterochromatin is mostly concentrated on centromeric regions of *A. velikensis* chromosomes. Obvious telomeric signals on some rod shaped bivalents as well as the X chromosome were also detected with $AgNO_3$ and DAPI staining. Although presented karyotype of *A. velikensis* resemble to those of other Helopini members and follows the common patterns of Tenebrionid karyotypes, slight differences in chromosome morphologies, NORs and the heterochromatin distribution were detected. Our specimens also showed a unique haplotype for COI sequences with an 84-83% sequence similarity to database sequences for Tenebrionidae.

Keywords. Karyotype, NOR, COI, DNA barcoding, Helopini, Tenebrionidae.

INTRODUCTION

Accanthopus Dejean, 1821 (= *Enoplopus* Solier, 1848) is a small tenebrionid genus with two lichen-feeding species, *A. velikensis* and *A. reitteri* (Brenske, 1884) distributed in Southern and partly Central Europe and occurring in *Fagus*, *Abies* and *Quercus* forests. Although the genus is considered to be included in the tribe Helopini since Lacordaire (1859), several additional taxonomic placements have been also proposed. Historically, the genus has been placed in either a separate tribe (*Enoplopites* – Solier 1848; Reitter 1917) or different subtribes in Helopini (i.e. Enoplopina – Reitter 1922; Nabozhenko 2018; Cylindrinotina – Ardoin 1958; Helopina – Nabozhenko 2008; Naboz-

henko and Löbl 2008). Ardoin (1958) also suggested erecting a separate tribe within the subfamily Tenebrioninae for this genus.

The genus *Accanthopus* has unusual external and internal structures, some of which support its position in the tribe Helopini. Several structures including the inner prothoracic skeleton, ovipositor, defensive glands female genital tubes are typical for Helopini (Tschinkel & Doyen 1980, Nabozhenko, 2005). On the other hand, the genus possess numerous non-helopine characters, such as very wide and spherical body, mentum with sexual dimorphism, profemora with strong and large acute tooth dorsally, strongly widened epipleura, very short and wide metaventrite, structures of mesonotum, metendosternite, aedeagus (Ardoin 1958), male inner sternite VIII and lobes of gastral spicula. Therefore, the position of *Accanthopus* in relation to other Helopini and Tenebrioninae lineages needs to be tested with additional data sets and integrated with molecular phylogenetic analyses.

The cytogenetic data among Tenebrionids have covered only about 1% of the species diversity (Guenin 1950, 1951a,b; Smith 1952; Smith and Virkki 1978; Yadav et al. 1980; Petitpierre et al. 1991; Juan and Petitpierre 1991a; Holecová et al. 2008; Blackmon and Jeffery 2015; Gregory 2016). In general, most of the species present a karyotype with 2n = 20, but the diploid number ranges from 2n = 14 to 2n = 38 in Tenebrionidae (Juan and Petitpierre 1991a; Pons 2004; Holecová et al. 2008; Lira-Neto et al. 2012). Based on available data, main karyological patterns in tenebrionid beetles were noticed in chromosome morphology, sex determining systems and distribution of heterochromatin (Juan and Petitpierre 1990; Petitpierre et al. 1991; Juan and Petitpierre 1991a, 1991b; Juan et al. 1993; Bruvo-Madaric et al. 2007; Şendoğan and Alpagut Keskin 2016).

Although chromosomal data are available for several representatives of subfamilies Allecullinae, Diaperinae, Lagriinae, Pimelinae, and Tenebrioninae, even basic information is scarce or totally lacking for other subfamilies (Juan and Petitpierre 1991a; Blackmon and Jeffery 2015). The chromosomes of *Accanthopus* have not yet been studied. Furthermore, cytogenetic data concerning the tribe Helopini are only known for some *Nesotes* Allard, 1876, *Euboeus* Boieldieu, 1865 (=*Probaticus* Seidlitz, 1896), *Nalassus* Mulsant, 1854 and *Turkonalassus* Keskin et al., 2017 species (Juan and Petitpierre 1986, 1989, 1991a, 1991b; Palmer and Petitpierre 1997; Şendoğan and Alpagut Keskin 2016).

Considering the limited cytogenetics information, the increase of chromosomal data may provide valuable phylogenetic signals about tenebrionid diversity. In this study, the mitotic and meiotic chromosomes of both sexes of *A. velikensis* were analysed using conventional and differential staining methods, with the aim of providing new data that will improve the knowledge on Tenebrionidae cytogenetics. We also sequenced the mt COI gene, for genetic identification of our *A. velikensis* specimens and barcoding of presented karyotype for further phylogenetic analysis.

MATERIALS AND METHODS

Specimens

Accanthopus velikensis specimens were collected from Pınarhisar, Kırklareli (41°46'02" N/27°37'51" E, 835 m). Adult beetles were collected on the trunks of trees at night when they are active.

Chromosome Analysis

Mitotic and meiotic chromosomes of 9 male and one female specimens were analysed using conventional and differential staining. Chromosome spreads were prepared from male and female gonads following the microspreading (Chandley et al. 1994) or splashing (Murakami and Imai 1974) methods with some modifications (Şendoğan and Alpagut Keskin 2016). The slides were stained with 4% Giemsa for 20 minutes for conventional staining. Silver impregnation technique of Patkin and Sorokin (1983) was performed to figure out the position of NOR regions. Chromosome spreads were examined and photographed with Zeiss Axio Scope A1 light microscope using ZEN software. The chromosomal measurements were obtained using the Levan plugin (Sakamoto and Zacaro 2009) and the karyotype was created with the CHIAS plugin (Kato et al. 2011) of the program Image J (Rasband 1997-2015). Heterochromatin distribution patterns were visualized with fluoroshield-DAPI (Sigma) specific to AT-rich chromosomal regions under Olympus BX50 fluorescent microscope.

mt COI barcoding

Genomic DNA was obtained from the thorax of the specimens using the Promega 96-well plate kit according to the manufacturer's instructions. The mitochondrial cytochrome oxidase I (COI) gene was amplified using the primers JerryTen and PatTen (Papadopoulou et al. 2009) for genetic identification of *A. velikensis* specimens and barcoding of the karyotype. PCR products

were purified and then sequenced in both directions. Sequencher 5.0 software was used to assemble and edit sequence chromatograms (Gene Codes, Ann Arbor, MI) and the COI sequences were submitted to GenBank for accession numbers. We performed a haplotype analysis using DnaSP v.5.10.1 (Rozas et al. 2017) and a BLAST search for all our sequences, in order to compare them with sequences deposited in GenBank.

RESULTS

We amplified the partial 829 bp sequences of cytochrome oxidase gene. Our specimens showed a unique haplotype for COI sequences with an 84-83% sequence similarity to database sequences for Tenebrionidae.

The cytogenetic analyses of spermatogonial and oogonial metaphase plates of *Accanthopus velikensis* revealed the diploid number to be 2n = 20, consisting of 2 pairs of metacentric and 7 pairs of submetacentric chromosomes (Figure 1-2, Table 1). While in male metaphase plates a minute subtelocentric *y* and a small submetacentric X chromosome appear as a heteromorphic pair (Figure 2b), no heteromorphism was observed among female metaphase plates (Figure 1a). X and *y* chromosomes are the smallest elements of the *A. velikensis* karyotype with the lengths of 2.434 µm and 0.759 µm, respectively (Table 1).

The observation of male metaphase I plates determined meioformula as 9 + Xy_p. The heteromorphic pair that composed the Xy_p was clearly observed in both conventionally and differentially stained male metaphase I plates (Figure 3a-c).

In diplotene/diakinesis of *A. velikensis*, 7 rod-shaped (terminal chiasma), and 3 ring-shaped (two terminal chiasmata) bivalents were observed (Figure 3d, 4d). In diakinesis/metaphase I; most of the homologous chromosomes

Table 1. Chromosome morphologies and measurements of *Accanthopus velikensis*. CI: centromere index, RL: relative length. (Centromere positions were determined according to Levan et al. 1964).

Chromosome	Length (µm)	%RL	CI	Morphology
1	4.999	13.7	45	M
2	4.466	12.2	28	SM
3	4.336	11.9	38	SM
4	3.771	10.3	28	SM
5	3.771	10.4	36	SM
6	3.553	9.8	30	SM
7	2.955	8.1	39	SM
8	2.782	7.6	44	SM
9	2.608	7.2	45	M
X	2.434	6.7	39	SM
y	0.759	2.1	23	ST

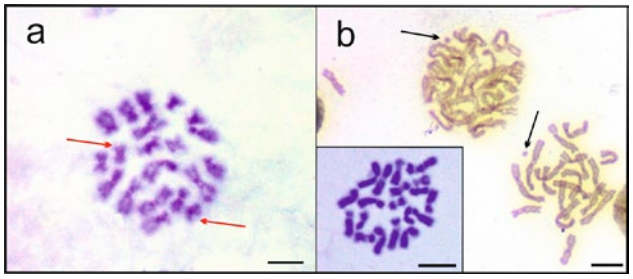


Fig. 1. (a) Oogonial metaphase (b) Spermatogonial metaphase of *Accanthopus velikensis*. Red and black arrows show X and minute y chromosomes respectively. Bars = 5µm.

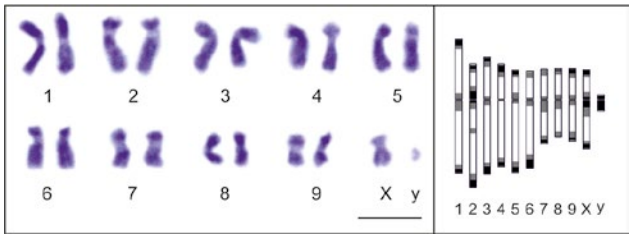


Fig. 2. Male karyotype and idiogram of *A. velikensis* 2n = 20. Bar = 5µm.

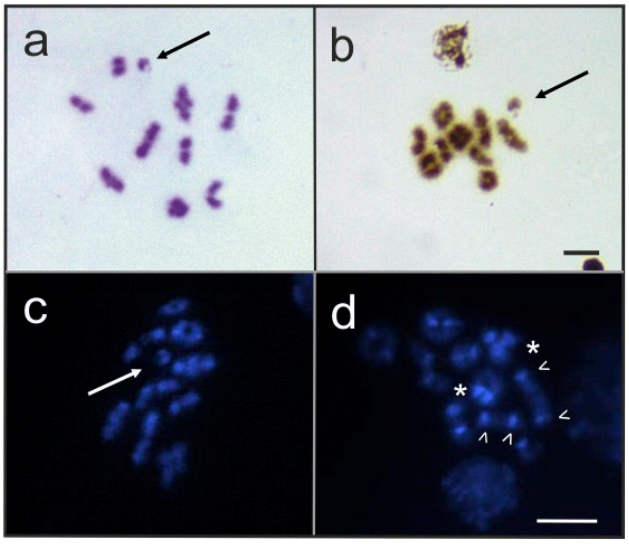


Fig. 3. Xy_p sex bivalents and heterochromatin in (a-c) Metaphase I, (a) Giemsa (b) Silver nitrate (c) DAPI staining (d) Diplotene-diakinesis (DAPI staining) Arrows show Xy_p sex bivalents, asterisk show heterochromatin. Arrowheads indicate telomeric signals on some of the rod shaped bivalents Bars = 5µm.

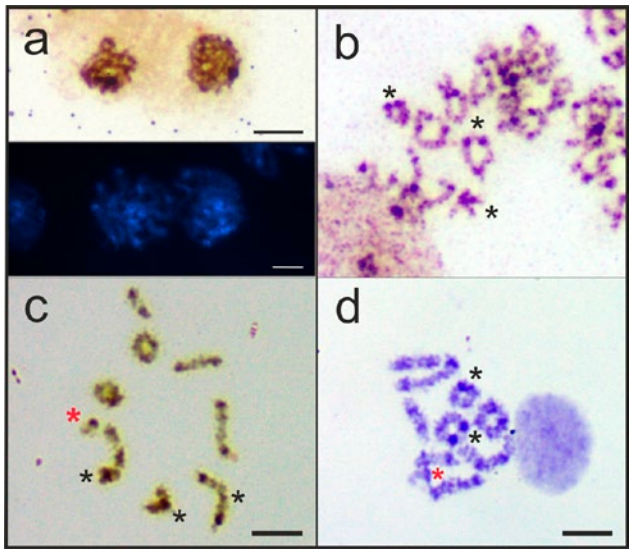


Fig. 4. NORs and heterochromatin in (a) Prophase I nuclei (silver and DAPI staining), (b) Diplotene-diakinesis, (c-d) pachytene after Silver (b-c) and Giemsa staining (d). Red asterisk indicate Xy_p and the presence of obvious signal in the long arm telomeric region of submetacentric X, black asterisks show differentially stained chromosome regions. Bars = 5μm (b-c possess the same scaling).

formed rod shaped bivalents and 1-2 cross-bivalents were also observed due to interstitial chiasma (Figure 3a–c).

In prophase nuclei, silver nitrate staining revealed the existence of a single impregnated mass of nucleolar material (Fig 4a). Additionally, obvious signals in the telomeric and pericentromeric regions of some autosomal pairs as well as Xy_p, were also observed in both silver nitrate and Giemsa stained diakinesis-metaphase I plates (Figure 4b-d). Giemsa staining of prophase nuclei indicated that all chromosomes of *A. velikensis* showed dark heterochromatic blocks mainly located in centromeric and pericentromeric regions (Figure 4d). Also with silver nitrate (Figure 4b-c) and DAPI staining (Figure 3d, 4a) rich telomeric and interstitial signals were observed in the large arms of most of the chromosomes.

In metaphase II plates, while some haploid sets seemed to be n = 9 due to minute *y* chromosome not being detectable (Figure 4b) the plates with the X chromosome showed the normal haploid number 10 (Figure 4a).

DISCUSSION

Due to predominant occurrence of the diploid number 2n=20 and parachute configuration of sex bivalents in the studied species, tenebrionid beetles considered as karyologically conservative group (Juan and Petitpierre 1988; 1991a; Juan et al., 1989; Palmer and Petitpierre

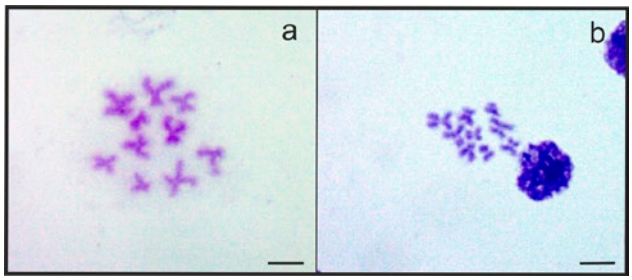


Fig. 5. (a.-b) Metaphase II plates (a) Haploid set with X chromosome (b) Haploid set with minute y chromosome which cannot be seen Bar = 5μm.

1997; Pons 2004). On the other hand, variations in sex chromosomes, NORs and heterochromatin distribution in spite of the shared modal number reveal that intrachromosomal rearrangements have played a major role in tenebrionid karyotype divergence (Juan et al. 1990; Almeida et al. 2000). The extent of diploid numbers between 14-38 within the family suggests that interchromosomal rearrangements such as Robertsonian processes or polyploidy could have also involved in karyotype evolution (Juan and Petitpierre 1991a; Petitpierre et al., 1991; Almeida et al. 2000; Pons 2004; Holecová et al. 2008; Lira-Neto et al. 2012; Goll et al. 2013).

We showed that the karyotype of *A. velikensis* consists of 10 pairs of chromosomes (2n=20, Xy_p) which are mostly submetacentric (Figure 1, 2, 3 a-c). This formula (n=10, Xy_p) was reported for other Helopini species as well i.e. *Nesotes* (Juan and Petitpierre 1986, 1989, 1991a), *Nalassus* and *Turkonalassus* (Şendoğan and Alpagut Keskin 2016). Despite this general resemblance, presence of mostly submetacentric chromosomes slightly differentiate *A. velikensis* karyotype from other Helopini possessing predominantly metacentric chromosomes. Furthermore, relative lengths of sex bivalents are obviously different in present karyotypes of Helopini. While X chromosomes of *A. velikensis* and *N. plebejus* (Küster, 1850) show similar relative lengths (6.9 % and 6.55 % respectively), *T. bozdagus* (Keskin et Nabozhenko, 2010) have clearly larger X (13.74 % of total complement) which has a conspicuous secondary constriction on the long arm. However, diploid numbers reported for the other helopine genera *Nesotes* (2n=20, Xy_p) and *Euboeus* (2n=20, XY) are based only on male metaphase I plates (Juan and Petitpierre 1986, 1989, 1991a, 1991b), and do not allow detailed comparison of chromosome morphologies.

Studies on differential patterns of karyotypes in Tenebrionidae and some other coleopteran families revealed the occurrence of heterochromatic blocks in mainly pericentromeric regions and autosomal or sex

chromosomal location of NORs (Juan and Petitpierre 1989; Juan et al. 1993; Pons 2004; Rozek et al. 2004; Schneider et al. 2007; Holecová et al. 2008; Karagyan et al. 2012; Goll et al 2013; Şendoğan and Alpagut Keskin 2016). The presence of heterochromatin blocks on pericentromeric regions of *A. velikensis* chromosomes was demonstrated with both $AgNO_3$ and DAPI staining (Figure 3d, 4). Additionally, telomeric signals on some rod shaped bivalents as well as the X chromosome (Fig 3d, 4 b-d) were detected. Our results showed that even a single NOR site was present in prophase I nuclei (Figure 4a), chromosomes in diakinesis-metaphase I plates gave multiple signals (Figure 4b-d). Therefore, further testing of exact NOR locations with rDNA-FISH probes is required to determine whether these signals are directly associated with NORs or a result of heterochromatin condensation.

In conclusion, karyotype of *A. velikensis* resemble those of other Helopini members and follows the common patterns of tenebrionid karyotypes with slight differences in chromosome morphologies, NORs and heterochromatin distribution. To truly understand these specific patterns of *A. velikensis* karyotype, comparative molecular cytogenetic studies with related taxa is required. In order to broaden the knowledge on the chromosomal evolution of tribe Helopini and assess the situation/position of *A. velikensis* within the tribe, cytogenetic studies should be combined with molecular phylogenetic analyses as well.

ACKNOWLEDGEMENTS

We are sincerely grateful to members of Molecular Cytogenetic Lab in Faculty of Medicine-Ege University for their help for fluorescent microscopy.

REFERENCES

Almeida MC, Zacaro AA, Cella DM. 2000. Cytogenetic analysis of *Epicauta atomaria* (Meloidae) and *Palembus dermestoides* (Tenebrionidae) with Xy sex determination system using standard staining, C-bands, NOR and synaptonemal complex microspreading techniques. Hereditas. 133: 147–157.

Ardoin P. 1958. Contribution a l'etude des Helopinae de France (Coleoptera, Tenebrionidae) [Contribution to the studies of Helopinae of France (Coleoptera, Tenebrionidae)] . Ann Soc Entomol Fr. 127: 9-49.

Blackmon H, Jeffery PD. 2015. Coleoptera karyotype database. Coleopt Bull. 69(1): 174–175.

Bruvo Madaric B, Plohl M, Ugarkovic D. 2007. Wide distribution of related satellite DNA families within the genus *Pimelia* (Tenebrionidae). Genetica. 130: 35–42.

Chandley AC, Speed RM, Ma K. 1994. Meiotic chromosome preparation. In: Gosden JR, editor. Chromosome analysis protocols. Methods in molecular biology. Humana Press; p. 27–40.

Goll LG, Artoni RF, Vicari MR, Nogaroto V, Petitpierre E, Almeida MC. 2013. Cytogenetic analysis of *Lagria villosa* (Coleoptera, Tenebrionidae): Emphasis on the mechanism of association of the Xy(p) sex chromosomes. Cytogenet Genome Res. 139(1): 29–35.

Gregory TR. 2016. Animal genome size database; [accessed: 2018 July 28]. http: //www.genomesize.com.

Guenin HA. 1950. Chromosomes et hétérochromosomes de ténébrionidés [Chromosomes and heterochromosomes of the Tenebrionids]. Genetica. 25: 157–182.

Guenin HA. 1951a. La formule chromosomiale de Coleopteres tenebrionides nordafricains, I. Pimelines et Tentyriines [The chromosomal formula of the North African Tenebrionid Coleopters, I. Pimeliini and Tentyriini]. Bull Soc Vaud Sci Nat. 65: 7–18.

Guenin HA. 1951b. La formule chromosomiale de Coleopteres tenebrionides nordafricains, II. Erodiines [The chromosomal formula of the North African Tenebrionid Coleopters, II. Erodiini]. Rev Suisse Zool. 58: 471–475.

Holecová M, Rozek M, Lachowska D. 2008. The first cytogenetic report on *Laena reitteri* Weise, 1877 with notes on karyotypes of darkling beetles. Folia Biol (Kraków). 56(3-4): 213–217.

Juan C, Petitpierre E. 1986. Karyological analyses on tenebrionid beetles from Balearic Islands. Genet Iber. 38: 231–244.

Juan C, Petitpierre E. 1988. A chromosome survey of North African and Eastern Mediterranean tenebrionids (Coleoptera). Cytobios. 54: 85–94.

Juan C, Petitpierre E. 1989. C-banding and DNA content in seven species of Tenebrionidae (Coleoptera). Genome. 32: 834–839.

Juan C, Petitpierre E, Oromi P 1989. Chromosomal Analyses on Tenebrionids from Canary Islands. Cytobios. 57: 33–41.

Juan C, Gosalvez J, Petitpierre E. 1990. Improving beetle karyotype analysis: Restriction endonuclease banding of *Tenebrio molitor* chromosomes. Heredity. 65: 157–162.

Juan C, Petitpierre E. 1990. Karyological differences among Tenebrionidae (Coleoptera). Genetica 80: 101–108.

Juan C, Petitpierre E. 1991a. Chromosome numbers and

sex determining systems in Tenebrionidae. In: Zunino M, Bellés X, Blas M, editors. Advances in Coleopterology. Barcelona: European association of coleopterology; p 67–176.

Juan C, Petitpierre E. 1991b. Evolution of genome size in darkling beetles. Genome. 34: 169–73.

Juan C, Pons J, Petitpierre E. 1993. Localization of tandemly repeated DNA sequences in beetle chromosomes by fluorescent in situ hybridization. Chromosome Res. 1: 167–174.

Kato S, Ohmido N, Fukui K. 2011. CHIAS 4 ver. 1.02.

Karagyan G, Lachowska D, Kalashian M. 2012. Karyotype analysis of four jewel-beetle species (Coleoptera, Buprestidae) detected by standard staining, C-banding, AgNOR-banding and CMA3/DAPI staining. Comparative Cytogenetics, 6(2): 183–197.

Lacordaire JT. 1859. Histoire naturelle des insectes, Genera des coléoptères, ou exposé méthodique et critique de tous les genres proposés jusqu'ici dans cet ordre d'insectes [Natural history of insects, Genera of coleopters, or methodical and critical exposition of all genera proposed so far in this order of insects]. Paris: Librairie Encyclopédique de Roret.

Levan A, Fredga K, Sonberg A. 1964. Nomenclature for centromeric position on chromosomes. Hereditas. 52: 201–220.

Lira Neto AC, Silva GM, Moura RC, Souza MJ. 2012. Cytogenetics of the darkling beetles *Zophobas aff. confusus* and *Nyctobates gigas* (Coleoptera, Tenebrionidae). Genet Mol Res. 11(3): 2432–2440.

Murakami A, Imai H. 1974. Cytological evidence for holocentric chromosomes of the silkworms, *Bombyx mori* and *B. mandarina*, (Bombycidae, Lepidoptera). Chromosoma 47: 167–178.

Nabozhenko MV. 2005. Interstructural correlations in evolution of darkling beetles of the tribe Helopini (Coleoptera: Tenebrionidae). Caucas Entomol Bull. 1(1): 37–48.

Nabozhenko MV. 2008. Tenebrionidae: Helopini. New nomenclatural and taxonomic acts and comments. In: Löbl I., Smetana A., editors. Catalogue of Palaearctic Vol 5 Coleoptera. Stenstrup: Apollo books; p. 36–38.

Nabozhenko MV. 2018. On the question of classification and phylogeny of the tribe Helopini Latreille, 1802 and resurrection of the subtribe Enoplopina Solier, 1848 (Coleoptera: Tenebrionidae). Caucas Entomol Bull. 14(2): 181-186.

Nabozhenko MV, Löbl I. 2008. Tribe Helopini. In: In: Löbl I., Smetana A., editors. Catalogue of Palaearctic Vol 5 Coleoptera. Stenstrup: Apollo books; p. 241–257.

Palmer M, Petitpierre E. 1997. New chromosomal findings on Tenebrionidae from Western Mediterranean. Caryologia. 50(2): 117–23.

Papadopoulou A, Anastasiou I, Keskin B, Vogler AP. 2009. Comparative phylogeography of tenebrionid beetles in the Aegean Archipelago: The effect of dispersal ability and habitat preference. Mol Ecol. 18: 2503–2517.

Patkin EL, Sorokin AV. 1983. Nucleolus organizing regions chromosomes in early embryogenesis of laboratory mice. Bull Exp Biol Med (USSR). 96: 92–94.

Petitpierre E, Juan C, Alvarez Fuster A. 1991. Evolution of chromosomes and genome size in Chrysomelidae and Tenebrionidae. In: Belles X, Blas M, Zunino M, editors. Advances in Coleopterology. Barcelona: European association of coleopterology; p. 129–44.

Pons J. 2004. Evolution of diploid chromosome number, sex-determining systems and heterochromatin in Western Mediterranean and Canarian species of the genus *Pimelia* (Coleoptera: Tenebrionidae). J Zool Syst Evol Res. 42: 81–85.

Rasband WS. 1997-2015. ImageJ. U.S. National institutes of health, Bethesda, Maryland, USA. http: //imagej.nih.gov/ij/

Reitter E. 1917. Bestimmungs schlüssel für die unterfamilien und tribus der paläarktischen Tenebrionidae [Determination key for the subfamilies and tribe of the Palearctic Tenebrionidae]. Wiener Entomologische Zeitung. 36: 51–66.

Reitter E. 1922. Bestimmungstabelle der paläarktischen Helopinae (Col. Tenebrionidae) I. teil [Determination table of the palearctic Helopinae (Col. Tenebrionidae) 1. part]. Wiener Entomologische Zeitung. 39: 1–44.

Rozas J, Ferrer Mata A., Sánchez DelBarrio JC, Guirao Rico S, Librado P, Ramos Onsins SE, Sánchez Gracia A. 2017. DnaSP 6: DNA sequence polymorphism analysis of large datasets. Mol Biol Evol. 34: 3299-3302.

Rozek M, Lachowska D, Petitpierre E, Holecová M. 2004. C-bands on chromosomes of 32 beetle species (Coleoptera: Elateridae, Cantharidae, Oedemeridae, Ceramcycidae, Anthicidae, Chrysomelidae, Attelabidae and Curculionidae). Hereditas. 140: 161–170.

Sakamoto Y, Zacaro AA. 2009. LEVAN, an ImajeJ plugin for morphological cytogenetic analysis of mitotic and meiotic chromosomes, Initial version. http: //rsbweb.nih.gov/ij/

Schneider MC, Rosa SP, Almeida MC, Costa C, Cella DM. 2007. Chromosomal similarities and differences among four Neotropical Elateridae (Conoderini and Pyrophorini) and other related species, with com-

ments on the NOR patterns in Coleoptera. J Zool Syst Evol Res. 45: 308–316.
Smith SG. 1952. The cytology of some tenebrionid beetles (Coleoptera). J Morphol. 91: 325–364.
Smith SG, Virkki N. 1978. Animal cytogenetics vol. 3 Insecta 5: Coleoptera. Berlin-Stuttgart: Borntraeger.
Solier AJJ. 1848 Essai sur les collaptérides [Studies on coleopters]. Studi Entomologici 1: 149–370.
Şendoğan D, Alpagut Keskin N**.** 2016. Karyotype and sex chromosome differentiation in two *Nalassus* species (Coleoptera, Tenebrionidae). Comp Cytogenet. 10(3): 371–385.
Tschinkel WR, Doyen JT. 1980. Comparative anatomy of the defensive glands, ovipositors and female genital tubes of Tenebrionid beetles (Coleoptera). Int J Insect Morphol Embryol. 9(5): 321-368.
Yadav JS, Pillai RK, Karamjeet. 1980. Chromosome numbers of Tenebrionidae (Polyphaga: Coleoptera). Biologia. 26: 31–41.

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: A. Teixeira Mesquita, M.V. Romero-da Cruz, A.L. Sousa Azevedo, E.R. Forni-Martins (2019) Chromosome number and genome size diversity in five Solanaceae genera. *Caryologia* 72(3): 105-115. doi: 10.13128/caryologia-772

Published: December 13, 2019

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

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

Chromosome number and genome size diversity in five Solanaceae genera

Amanda Teixeira Mesquita[1,*], Marìa Victoria Romero-da Cruz[1], Ana Luisa Sousa Azevedo[2], Eliana Regina Forni-Martins[1]

[1] *Departamento de Biologia Vegetal, Universidade Estadual de Campinas, Rua Monteiro Lobato 255, CEP: 13.083-970 Campinas (SP), Brasil*
[2] *Embrapa Gado de Leite, Empresa Brasileira de Pesquisa Agropecuária (Embrapa), Rua Eugênio do Nascimento 610, CEP: 36.038-330 Juiz de Fora (MG), Brasil*
*Corresponding author: mesquita.at@gmail.com

Abstract. Sixteen species of Solanaceae, belonging to five genera, were studied karyologically through chromosome counting, chromosomal measurement, and karyotype symmetry. Genome size (GS) estimation was performed on fifteen species using flow cytometry. The chromosome number 2n=24 was found in all *Solanum* species and *Acnistus arborescens*, 2n=22 was found in *Brunfelsia uniflora,* and 2n=16 in *Cestrum* representatives. *Physalis pubescens* was the only specie with evidence of polyploidy, showing 2n=4x=48 chromosomes. The chromosome numbers of *S. adspersum*, *S. inodorum*, *S. flaccidum, S. sanctae-catharinae, and B. uniflora* were reported for the first time. Haploid karyotype length (HKL) was statistically different between the studied species. The polyploid *P. pubescens* showed the largest HKL value, 93.10 μm. In general, karyotypes were symmetrical with predominance of metacentric chromosomes. Chromosome size was small in most species (<4 μm), while *S. diploconos*, *C. laevigatum,* and *C. mariquitense*, species with high HKL values, exhibited larger chromosomes. Genome size estimation were unpublished for ten studied species and were the first estimation for the genera *Acnistus*, *Brunfelsia* and *Physalis*. Were observed about eight-fold differences between species with averages varying from 2C=2.57 pg to 2C=20.27 pg. As both HKL and GS showed a continuous variation. We observed partial similarity in the species ordered according to HKL and GS. The Solanaceae genera showed a constant chromosome number and a tendency to posse symmetrical karyotypes. The genome size also showed differences, which suggests that chromosome evolution in the group could be driven by alterations in the repetitive fractions of the genome.

Keywords. *Acnistus*, *Brunfelsia*, *Cestrum*, *Physalis*, *Solanum*, karyotype evolution.

INTRODUCTION

The Solanaceae family comprises about 2,500 species and 100 genera and have cosmopolitan distribution. The greatest diversity of the family is found in Neotropical regions (D'Arcy 1991; Hunziker 2001). Members of Solanaceae have great ecological and morphological diversity, characteristics which favoured the occupation of diverse habitats, such as desert regions, tropi-

cal rainforests, and even disturbed areas (D'Arcy 1991; Knapp 2002).

The family includes several species of important global food crops with high economic value, such as tomatoes (*Solanum lycopersicum*), potatoes (*Solanum tuberosum*), eggplants (*Solanum melongena*), and chilli peppers (*Capsicum* spp.), widely used drug plants, such as tobacco (*Nicotiana tabacum*), "datura" (*Datura stramonium*), and "angel's tears" *Brugmansia suaveolens*, as well as many ornamental plants, such as species of the genus *Brunfelsia*, *Cestrum* and *Petunia*. Many Solanaceae species, including tomatoes, potatoes, and tobacco, are model organisms for various biological studies, and their genomes are some of the most well studied among angiosperms (Knapp et al. 2004).

Karyotype information about species and groups are important for taxonomic and evolutionary studies, whereas karyological changes accompany speciation and, consequently, the diversification of the groups (Guerra et al. 2008, 2012, Chiarini et al. 2018). The chromosome number, nuclear DNA content, total length of the chromosome complement, asymmetry indices, and number and location of the rDNA sites and heterochromatic bands are the main data used in cytotaxonomic studies. Chromosome number data is the most available information and is not influenced by external agents, such as age of individuals, environmental conditions, and gene expression, providing accurate data about species evolution (Dobginy et al. 2004, Guerra et al. 2008, 2012). Cytogenetic characterization, accompanied by a genome size (GS) study, can offer important information about genome organization, phylogenetic relationships, and evolutionary trends. This approach has been successful used in some Solanaceae (Mishiba et al. 2000, Moscone 2003, Chiarini et al. 2014).

Chromosome data is available for some genera of Solanaceae, while for other genera there is not enough data or information about their chromosomes. *Lycium* and *Solanum* present constant chromosome number (2n=24 and polyploids) (Bernadello and Anderson 1990; Bernadello et al. 1994; Chiarini and Bernadello 2006; Rego et al. 2009; Stiefkens et al. 2010; Melo et al. 2011; Chiarini et al. 2014), while *Capsicum* shows 2n=24 and 2n=26 (Moscone 1993; Moscone et al. 2007; Aguilera et al. 2014; Grabiele et al. 2014; Romero-da Cruz and Forni-Martins 2015; Romero-da Cruz et al. 2017). For the Cestreae tribe, composed of *Cestrum*, *Sessea,* and *Vestia*, the only chromosome number reported to date is 2n=16 (Fregonezi et al. 2006; Las Peñas et al. 2006; Fernandes et al. 2009; Urdampilleta et al. 2015). The greatest range in chromosome number is found in *Nicotiana* (n=12 to n=32, and polyploids, Chase et al. 2003).

Only about 8% of Solanaceae taxa have available GS data. This character has more variability than chromosome number (Soltis et al. 2003). In *Solanum*, the GS ranges of from forty-fold in species with 2n=24 chromosomes. The smallest reported C-value is in *S. chacoense*, 1C=0.63 pg (Bennett and Smith 1976), while the largest value is 1C= 24.80 pg, found in *S. hartwegii* (Pringle and Murray 1991).

Nevertheless, there are still many gaps in karyotypic knowledge for the Solanaceae family and such information (i.e. genome size, chromosome number, and karyotype variables) is important to complete current data and to better understand the systematic relationships and chromosome evolution of the family. Therefore, the objectives of this study were: (1) to report original chromosome numbers and describe the karyotype variables in distinct genera of the Solanaceae family, (2) to determine the genome size (GS) using flow cytometry for the first time for many species.

MATERIAL AND METHODS

Plant material

Sixteen species from the genera *Acnistus*, *Brunfelsia*, *Cestrum*, *Solanum,* and *Physalis* were collected in Southeastern Brazil. Voucher specimens were deposited into the Herbarium at the University of Campinas (UEC). Data collection is detailed in Table 1.

Chromosome preparations

Seeds of at least three individuals per species were germinated in Petri dishes. In some cases, 1 ml gibberellic acid (GA_3) was applied to break seed dormancy (Ellis et al. 1985). According to previous tests, root meristems were pre-treated with different solutions to block the cell cycle to obtain good chromosome spread and condensation (Table 2). The root apices were fixed in 3:1 ethanol: acetic acid (v:v) mixture that was stirred for a minimum of 12 h at room temperature (RT) and stored at -6ºC until slide preparation. Slides were made using root meristems that were previously digested in a solution of 1% macerozima, 2% cellulase, and 20% pectinase for 10-15 minutes at 37ºC and squashed in a drop of 45% acetic acid. Coverslips were removed after freezing in liquid nitrogen for 15 minutes. The cells were photographed under a microscope Olympus BX51 with a DP72 camera attached and images were captured using Olympus DP2 BSW program (Olympus Corporation).

Table 1. Cytogenetics data of Solanaceae species: Species and voucher specimen; provenance of materials; chromosome number, haploid karyotype formula (HKF), median haploid karyotype length (HKL), variation in chromosome length (VCL); symmetry indices A1 and A2; median DNA content (2C values).

Species (Voucher specimen)	Provenance	2n	HKF	HKL – µm (CI)	VCL – µm	A1	A2	2C values – pg (CI)
Acnistus arborescens Schltdl. (Monge 2787)	Brazil: Rio Grande do Sul; Aratinga	24	12m	45.93 (2.78)	3.17-4.38	0.17	0.10	6.56 (0.06)
Brunfelsia. uniflora D. Don (Mesquita 15)	Brazil; São Paulo; Campinas	22	7m+4sm	50.51 (0.50)	3.89-5.37	0.32	0.10	6.58 (0.13)
Cestrum laevigatum Schltdl. (Mesquita 12)	Brazil; São Paulo; Campinas	16	6m+2sm	78.72 (2.96)	7.92-10.88	0.23	0.10	20.27 (0.43)
C. mariquitense Kunth (Mesquita 14)	Brazil; São Paulo; Campinas	16	7m+1sm	73.91 (6.38)	7.35-11.39	0.21	0.12	-
Physalis pubescens L. (Vasconcellos Neto 00-068)	Brazil; São Paulo; Jundiaí	48	19m+5sm	93.10 (2.87)	1.43-2.80	0.32	0.18	12.98 (0.09)
Solanum								
Cyphomandra clade								
Solanum diploconos (Mart.) Bohs (Mesquita 23)	Brazil; São Paulo; Jundiaí	24	8m+4sm	74.72 (1.86)	4.63-7.49	0.32	0.14	19.22 (0.43)
Dulcamaroid clade								
S. flaccidum Vell. (Mesquita 07)	Brazil; São Paulo; Campinas	24	9m+2sm+1st	26.73 (1.14)	1.83-2.50	0.27	0.10	2.57 (0.25)
S. inodorum Vell. (Vasconcellos Neto 20401)	Brazil; São Paulo; Jundiaí	24	5m+7sm	38.33 (3.90)	2.83-3.86	0.39	0.09	4.63 (0.06)
Geminata clade								
S. pseudocapsicum L. (Mesquita 24)	Brazil; São Paulo; Jundiaí	24	9m+3sm	28.61 (7.70)	1.76-2.72	0.28	0.13	2.94 (0.11)
Leptostemonum clade								
***Acanthophora* section**								
S. acerifolium Sendt. (Mesquita 02)	Brazil; São Paulo; Campinas	24	10m+2sm	36.17 (1.09)	1.71-3.87	0.26	0.23	5.69 (0.15)
S. palinacanthum Dunal (Mesquita 20)	Brazil; São Paulo; Ubatuba	24	5m+7sm	37.86 (0.72)	2.51-3.86	0.41	0.13	5.00 (0.10)
***Torva* section**								
S. adspersum Witasek (Monge 2748 c 240)	Brazil; Rio de Janeiro; Arraial do Cabo	24	9m+3sm	25.09 (1.62)	1.77-2.44	0.25	0.09	3.19 (0.04)
S. scuticum M. Nee (Vasconcellos Neto 8503)	Brazil; São Paulo; Jundiaí	24	9m+3sm	27.66 (2.10)	1.95-2.79	0.31	0.04	3.42 (0.06)
S. variabile Mart (Monge 2324)	Brazil; São Paulo; Itacaré	24	9m+3sm	33.45 (0.51)	2.15-3.26	0.24	0.11	3.54 (0.09)
Uncertain position								
S. concinnum Schott ex Sendtn. (Mesquita 08)	Brazil; São Paulo; Campinas	24	6m+6sm	31.82 (0.37)	2.26-2.86	0.39	0.09	3.65 (0.25)
S. sanctae-catharinae Dunal (Vasconcellos Neto 20873)	Brazil; São Paulo; Jundiaí	24	10m+2sm	23.15(2.56)	1.68-2.35	0.26	0.09	3.79 (0.07)

CI – Confidence interval at 95% of semi range.

Karyotype analysis

Five metaphases of each species, with the same degree of chromosome condensation, were used to determine the chromosome number. The measurements were taken using the MicroMeasure© software (3.3). Ideograms were made using measurements of the following means for each chromosome pair: S (short arm length), L (long arm length) and C (total chromosome length) using the formula C= S+L. In addition, haploid karyotype length (HKL) was calculated by the sum of the haploid chromosome lengths. The arm ratio (r) was calculated using the formula r= L/S and was used to classify chromosomes according to Levan et al. (1964). For ideograms, chromosomes were first grouped by morphology (r=1.00-1.69 metacentric-m; r=1.70-2.99 submetacentric-sm; r=3.00-6.99 subtelocentric-sm) and then by decreasing size order within each group.

The karyotype symmetry was described using the indices A1= 1-[(Σbi/Bi)/n] (bi = mean of the short arm

of each chromosome pair, Bi = average of the long arm of each chromosome pair, n = number of chromosome pairs) and A2=x/s (s = standard deviation; x = average chromosome complement length) (Zarco 1986). A1 index measures intrachromosomal asymmetry which indicates differences in the size of chromosome arms. A2 index measures the interchromosomal asymmetry and indicates the variation in chromosome lengths. In terms of length, chromosomes were classified according to Lima de Faria (1980) as very small (≤1 μm), small (>1 μm and ≤4 μm), intermediate (>4 and ≤12) and big (>12 and ≤60).

Flow cytometry

The same species that were cytogenetically analysed (except for *Cestrum mariquitense*) were cultivated in a greenhouse and used for GS measurements. For each species, three individuals were measured in three repetitions, for a total of nine samples. Approximately 1 cm^2 of young leaf tissue was used to prepare the nuclear suspensions, according to Dolezel et al. (2007). The material of each species of interest and a piece of internal leaf standard (*Pisum sativum* "Ctirad" 2C=9.09 pg (Dolezel et al. 1998) were sliced with a razor blade and placed into a Petri dish on ice. About 1 ml of LB01 buffer (Dolezel et al. 1989) was used to extract the nuclei. A nylon mesh with 40 microns was used to filter the sample (CellTrics, PARTEC), then, 25 μL 1 mg/mL propidium iodide and 25 μL 1 mg/mL RNAse were added to the nuclear suspension. The measurement was performed on a BD FACS Calibur flow cytometer, for each sample an average of 10,000 nuclei were analysed. The 2C value was calculated using the linear relationship between fluorescence signals from stained nuclei of the unknown sample and the reference standard. The nomenclature for genome size classification followed Leitch et al. (1998) with modification by Soltis et al. (2003): values <1.4 pg and between 1.4 to 3.5 pg correspond to "very small" and "small" genomes, respectively. On the other hand, values between 3.51-13.99 pg, >14 pg and >35 pg are considered "intermediate," "large", and "very large" genomes, respectively.

Statistical analyses

The HKL values, as well of GS values of each species, were compared using Past 3.18 ® (Øyvind Hammer, Natural History Museum, University of Oslo). The Kruskal-Wallis nonparametric test was performed to compare the averages among the species and Dunn's post-hoc test (Dunn 1954) was carried out after significant Kruskal-Wallis test.

RESULTS

Karyotype analysis

The somatic chromosome numbers were 2n=2x=24 (*Acnistus* and *Solanum*), 2n=2x=22 (*Brunfelsia*), 2n=2x=16 (*Cestrum*) and 2n=4x=48 (*Physalis*) (Table 1; Fig. 1).

Although the differences in HKL between some species were significant ($p<0.05$) according to statistical analysis (Table 1; Fig. 2), this variation was gradual, and no groups were formed. *Solanum sanctae-catharinae* showed the lowest median value (23.15 μm) with a variation of 1.68-2.35 μm from the smallest to largest chromosome pair. Other *Solanum* species also presented low HKL (except for *S. diploconos*), with values reaching 38.33 μm in *S. inodorum* (2.83 to 3.86 μm). Species with intermediate HKL values were *A. arborescens* (45.93 μm, 3.17 to 4.38 μm) and *B. uniflora* (50.51 μm, 3.89 to 5.38 μm). High HKL values were found in *C. mariquitense* with 73.91 μm (7.35 to 11.39 μm), *S. diploconos* with 74.72 μm (4.63 to 7.49 μm), and *C. laevigatum* with 78.72 μm (7.92 to 10.88 μm). *Physalis pubescens* showed

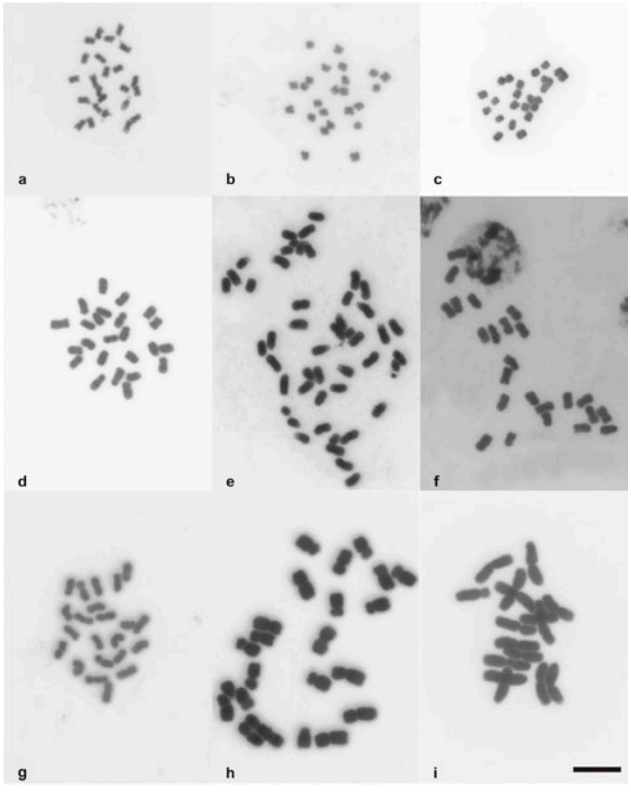


Fig. 1. Somatic metaphases of five genera of Solanaceae. **a** *Solanum flaccidum*. **b** *S. adspersum*. **c** *S. sanctae-catharinae*. **d** *S. inodorum*. **e** *Physalis pubescens*. **f** *Acnistus arborescens*. **g** *Brunfelsia uniflora*. **h** *S. diploconos*. **i** *Cestrum laevigatum*. Bar=10 μm.

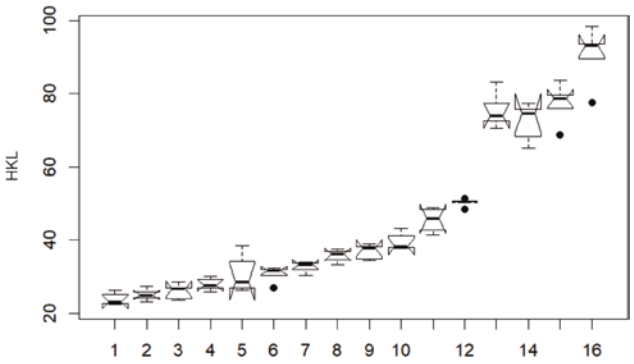


Fig. 2. Boxplots illustrating the continuous variability of HKL (Haploid Karyotype Length), as inferred from de Kruskal Wallis analysis. The numbers on the x axis represent the species ordered by crescent HKL values (in µm): *S. sanctae-catharinae* (1), *S. adspersum* (2), *S. flaccidum* (3), *S. scuticum* (4), *S. pseudocapsicum* (5), *S. concinnum* (6), *S. variabile* (7) *S. acerifoium* (8) *S. palinacanthum* (9) *S. inodorum* (10), *A. arborescens* (11) *B. uniflora* (12) *C. mariquitense* (13) *S. diploconos* (14) *C. laevigatum* (15) *P. pubescens* (16). The central box represents 50% of the data from de upper to lower quartile. The horizontal bar expresses the median position. The extremity of the vertical lines indicates minimum and maximum values of HKL, if they are no outliners. When outliners are present, they are represented by circles.

the highest HKL value (93.10 µm), even though it is a polyploid species with chromosomes ranging from 1.43 to 2.8 µm.

Karyotypes are symmetrical with A1 and A2 values for each species ranging from 0.17 to 0.41 and from 0.04 to 0.23, respectively. Most species presented a predominance of metacentric chromosomes (Table 1, Fig. 3) that characterized most intrachromosomal symmetry shown in the A1 index. *Acnistus arborescens* had the most symmetrical karyotype, composed of only metacentric chromosomes and A1=0.17. Three species had less symmetrical karyotypes: *Solanum inodorum* and *S. palinacanthum* showed predominance of submetacentric chromosomes (5m+7sm) and A1 value of 0.39 and 0.41, respectively. *Solanum concinnum* also presented A1=0.39, but karyotype formulae 6m+6sm.

Interchromosomal index A2 showed that all species have few variations in chromosome size of the karyotypes. *Solanum scuticum* showed the small A2 value (0.04) and *Solanum acerifolium* presented the highest A2 value (0.23), characterizing the most interchromosomal asymmetry among studied species (Table 1).

C-value

Genome size estimates of all the studied species are shown in Table 1 and histograms for selected species are shown in Fig. 4. According to statistical analysis, GS showed significant differences among some of the studied species (Fig. 5). A variation of about eight-fold was observed, ranging from 2C=2.57 pg (*S. flaccidum*, Fig. 4a) to 2C=20.27 pg (*C. laevigatum*, Fig. 4d). The GS presented continuous variation, so distinct groups were not characterized (Fig. 5). Most species had small (2C=2.57 pg in *S. flacidum* to 2n=3.79 pg in *S. sanctae-catharinae*) and intermediate genomes (2C=4.63 pg in *S. inodorum* to 6.56 pg in *A. arborescens* and 6.58 pg in *B. uniflora*). The species with larger genomes were *P. pubescens* (2n=12.98 pg), *S. diploconos* (2C=19.22 pg), and *C. laevigatum* (2C=20.27 pg).

DISCUSSION

Chromosome number

The chromosome number data found here are new for *S. adspersum*, *S. inodorum*, *S. flaccidum,* and *S. sanctae-catharinae*, with 2n=24 chromosomes, as well as for *B. uniflora*, with 2n=22. For the remaining species, the chromosome number obtained corroborated with data found in the literature for *Acnistus* (2n=24), *Cestrum* (2n=16), *Solanum* (2n=24), and *Physalis* (2n=48) (Heiser 1963; Pedrosa et al. 1999; Fernandes et al. 2009; Rego et al. 2009; Urdampilleta et al. 2015).

All the species in this study, except for *P. pubescens*, which is a tetraploid, are diploid. Although diploid is the most frequent ploidy level (including other species of *Physalis*), polyploidization has played an important role in the evolution of some Solanaceae genera (e.g., *Nicotiana*, Chase et al. 2003; *S. elaeagnifolium*, Scaldaferro et al. 2012). The chromosome number most frequent in the family is 2n=24, found in more than 85% of the previously studied Solanaceae species (Olmstead et al. 2008) though a diploid series from 2n=14 to 2n=26 is present in some genera (eg. *Petunia* and *Calibrachoa*, Mishiba et al. 2000, *Cestrum, Sessea and Vestia,* Las Peñas et al. 2006, *Capsicum*, Moscone et al. 2007).

Many authors have postulated hypotheses for the ancestral chromosome base number in the family. Raven (1975) proposed x=7 and 12 for the order Solanales and Solanaceae family, respectively, while Badr et al. (1997) suggested the hypothesis of x=7 or x=8. Moscone (1992) corroborate with the proposition of Badr et al. (1997), suggested x=7 as the basic chromosome number for Solanaceae. Olmstead and Palmer (1992) and Olmstead et al (2008) based in phylogenetic studies, suggests an ancestral position of subfam. Cestroideae (x=8), and x=12 as a derivate basic chromosome number the family.

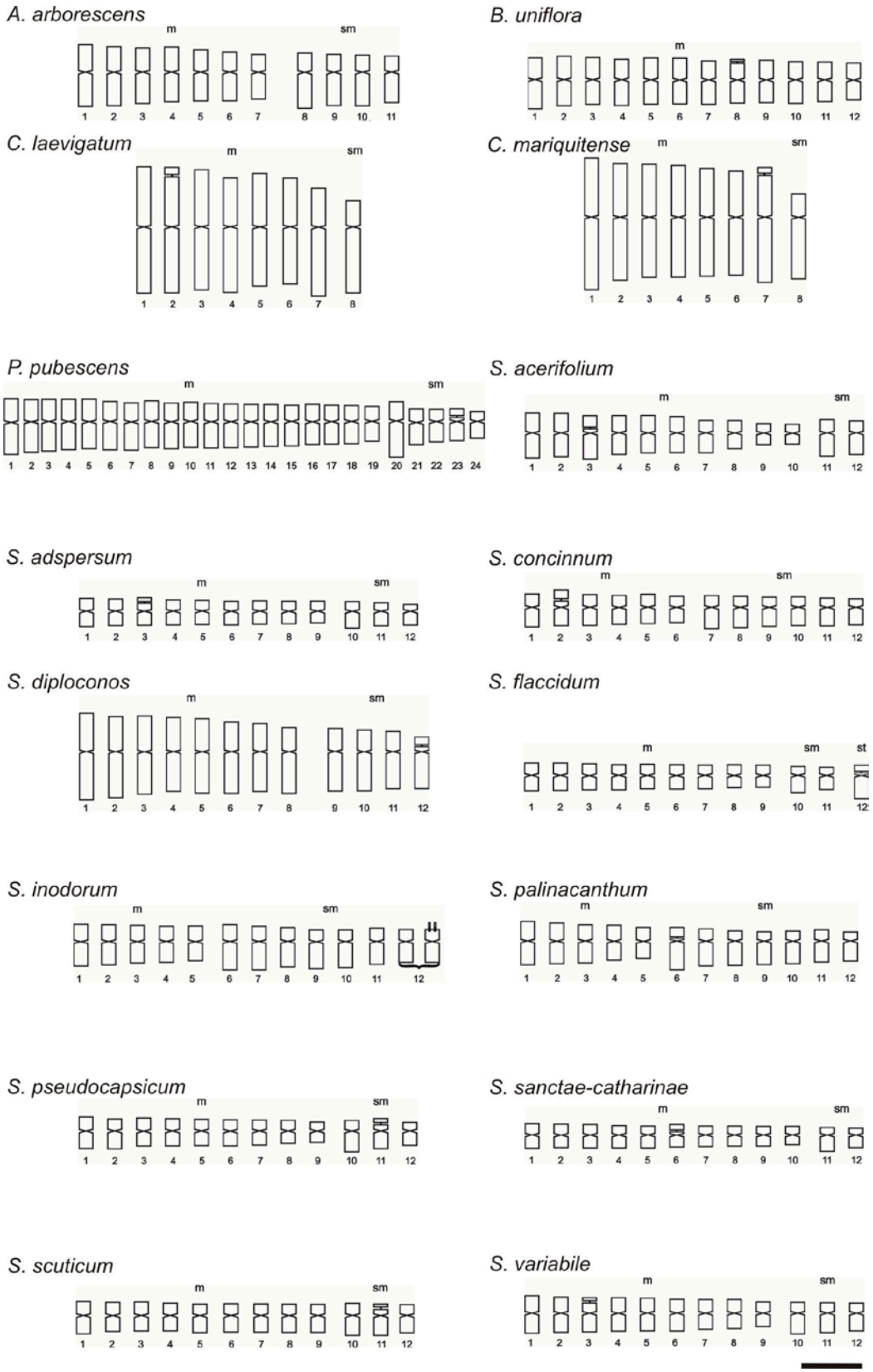


Fig. 3. Ideograms of the investigated Solanaceae species based on median chromosome values. Bar=5 μm.

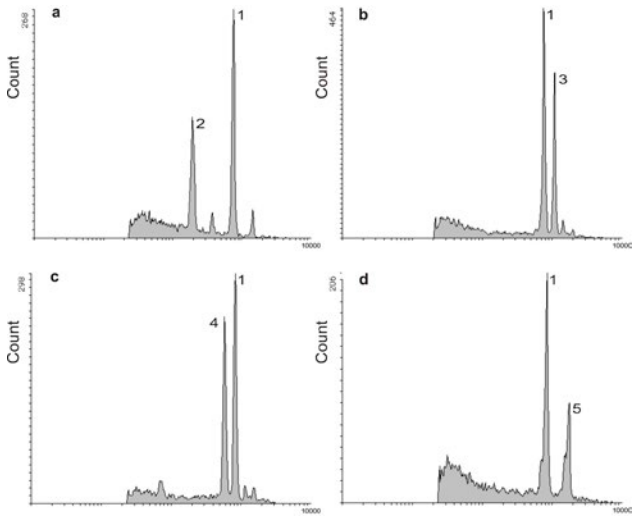


Fig. 4. Flow cytometry histograms (iodide propidium fluorescence intensity of nuclei) showing DNA amounts from leaf tissues of some Solanaceae species. **1** *Pisum sativum* "Ctirad" (standard). 2 *S. flaccidum*. **3** *P. pubescens*. **4** *A. arborescens*. **5** *C. laevigatum*.

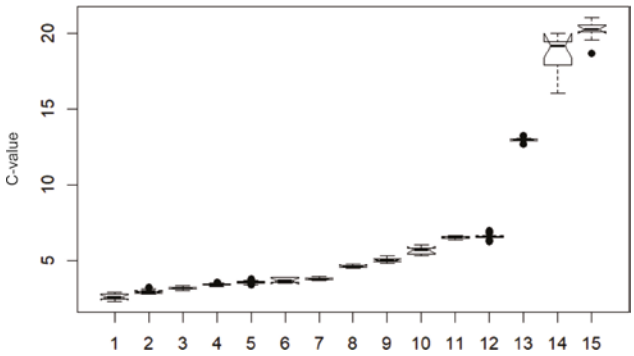


Fig. 5. Boxplots illustrating the continuous variability of GS (Genome Size), as inferred from de Kruskal Wallis analysis. The numbers on the x axis represent the species ordered by crescent C-values (in pg): *S. flaccidum* (1), *S. pseudocapsicum* (2), *S. adspersum* (3), *S. scuticum* (4), *S. variabile* (5), *S. concinnum* (6), *S. sanctae-catharinae* (7) *S. inodorum* (8) *S. palinacanthum* (9) *S. acerifolium* (10), *A. arborescens* (11) *B. uniflora* (12) *P. pubescens* (13) *S. diploconos* (14) *C. laevigatum* (15). The central box represents 50% of the data from de upper to lower quartile. The horizontal bar expresses the median position. The extremity of the vertical lines indicates minimum and maximum values of HKL, if they are no outliners. When outliners are present, they are represented by circles.

The lack of chromosomal data for several genera and for the Solanaceae sister group, the family Convolvulaceae, as well as the presence of distinct basic numbers in other Solanales families, as in Hydroleaceae, x=8 and 10 (Constance 1963) and Montiniaceae, x=12 (Goldblatt 1979), has hampered to establish a consensus about a basic chromosome number and understand the direction of chromosome number evolution for the family.

Karyotype structure

Differences in chromosome size were seen between the Solanaceae species here investigated.

The relatively small chromosome size and HKL observed in the species here of *Solanum*, except for *S. diploconos* (statistically distinct, and previously considered a species of the distinct genus *Cyphomandra*), and *P. pubescens*, have been reported in some studies for *Solanum* (Bernardello and Anderson 1990; Acosta et al. 2005; Chiarini et al. 2006; Rego et al. 2009; Melo et al. 2011; Moyetta et al. 2013), and another Solanaceae genera, as *Lycianthes* and *Vassobia* (Rego et al. 2009) and *Lycium*, (Stiefkens and Bernadello 2002,)

Acnistus arborescens and *B. uniflora* shows chromosomes and consequently, HKL values, with intermediate size, when compared to *Solanum* and *Physalis*. These karyotype characteristics are also present in *Capsicum* (Moscone 1996), *Sclerophylax* and *Nolana* (Lujea and Chiarini 2017), genera also belonging to Solanaceae. Although the intermediate size of the chromosomes, a constant chromosome number, karyotype symmetry and chromosomes majority metacentric appear to maintain in these groups.

The tribe Cestreae (subfam. Cestroideae) embraces the genera *Cestrum*, *Sessea* and *Vestia*, presents the largest chromosomal sizes of the family (Fregonezi et al. 2006, Peñas et al. 2006). *Cestrum laevigatum* and *C. mariquitense,* investigated here, showed the largest chromosome size high HKL values, confirming the trend for the tr. Cestreae (Fregonezi et al. 2006). Such increase in chromosome size for the tribe can be due to the absence of *Arabidopsis*-type telomeres $(TTTAGGG)_n$ for short interstitial telomeric sequences (SITS), leading to the lack of control of the telomerase-dependent replication. These sequences may associate with other DNA sequenc-

Table 2. Pretreatments used for each genus of Solanaceae studied.

Genus	Pretreatments
Acnistus and *Physalis*	8-hydroxyquinoline 0.002M + cycloheximide 25mg/L (1:1), 8 h, 4ºC
Brunfelsia	8-hydroxyquinoline 0.002M, 6 h, 14ºC
Cestrum	Colchicine 0.1% 6 h, RT*
Solanum	Saturated solution of r-dichlorobenzene 2 h, RT*
Solanum (*S. diploconos*)	Saturated solution of r-dichlorobenzene 5 h, RT*

*RT=room temperature.

es and assist in their dispersion leading to an increase in genome size (Sykorova et al. 2003a, b).

Besides chromosome number, other widely conserved karyotype characters in Solanaceae genera, are chromosome morphology and karyotype symmetry. Symmetrical karyotypes with a predominance of metacentric chromosome pairs are found in the five genera studied here. *Acnistus arborescens* was the unique specie that had only metacentric chromosomes, thus, had the most symmetrical karyotype and only *S. flaccidum* showed a subtelocentric chromosome pair. Other genera of the family Solanaceae in which it is possible to observe these characteristics are*Capsicum* (Pozzobon et al. 2006; Moscone et al. 1993, 2007), *Lycium* (Stiefkens and Bernadello 2012), *Lycianthes,* and *Vassobia* (Rego et al. 2009).

Among the angiosperms, karyotype asymmetry can be associated with derivate taxa (Stebbins 1971) In some groups of the Solanaceae family, intermediate asymmetry values, can be observed, such as the tr. Cestreae (Las Peñas et al. 2006), *Solanum* sect. *Acanthophora* (Chiarini et al. 2014) However, for species, the karytotype asymmetry was not associate with basal or derived position of the taxa in the phylogeny. Regarding karyotype asymmetry, no evolutive trend was found for the analysed genera or among the representatives of *Solanum*. Overall, karyotype asymmetry seems to occur randomly within some groups of the family.

Our study analysed five species from other sections (*Acantophora* and *Torva*) of the Leptostemonum clade. In sect. *Acantophora* (*S. acerifolium* and *S. palinacanthum*), we observed greater HKL and karyotype asymmetry than in sect. *Torva* (*S. adspersum*, *S. scuticum* and *S. variabile*). Karyotype asymmetry was previously reported for the Lepstotemomun clade (Chiarini et al. 2014). In other cases, asymmetry was random within a group, as in *Solanum* Morelloid and Dulcamaroid clades (Moyetta et al. 2013). Both species belonging to clade Dulcamaroid that were studied corroborated this data, while *S. inodorum* (HKL=38.33 µm) presented a more asymmetrical karyotype (5m + 7sm and A1=0.39, A2=0.09) than *S. flaccidum* (HKL=26.73 µm, 9m + 2sm + 1st and A1=0.27, A2=0.10).

The karyotype characteristics described for the studied species and genera as well as in other Solanaceae groups, a constancy in the chromosome number, karyotype symmetry and chromosome morphology, indicates karyotype orthoselection, which preserves similar chromosomal complements, regardless of chromosome size (Acosta et al. 2005; Moscone et al. 2003). According to Wu and Tanksley (2010), inversions have occurred at a much higher rate than translocations throughout the evolutionary history of Solanaceae, thereby preserving chromosome morphology favouring chromosomal uniformity

C-value/DNA content

Despite the great number of representatives in Solanaceae, the GS estimation is available for a small proportion of species and genera. Only 12 Solanaceae genera have data about GS, representing approximately 10% and 186 species that corresponding to 7% of Solanaceae representatives. Genome size data for *A. arborescens*, *B. uniflora* and *P. pubescens* are the first estimation for the relative genera. Some species of *Cestrum* and *Solanum* have their GS measured but the data here obtained are unpublished for *C. laevigatum*, *S. flaccidum*, *S. inodorum*, *S, adspersum*, *S, scuticum*, *S. variabile*, *S. concinnum* and *S. sanctae-catharinae*.

The GS variation observed in the species studied partially coincides with the variation observed in HKL. In general, species with small, intermediary, or high HKL presented the same GS classification. Among the five species with small HKL, four presented small values of DNA content (*S. adspersum*, *S. flaccidum, S. pseudocapsicum* and *S. scuticum*). Similarly, of the six species with high HKL, five showed high values of DNA content (*A. arborescens*, *B. uniflora*, *S. diploconos*, *C. laevigatum* and *P. pubescens*). The estimation of nuclear DNA using flow cytometry is more accurate than measuring chromosomes. This accuracy is supported by statistical tests and by species boxplots, where the dispersion of HKL data (Figure 2) was greater than GS data (Figure 5). Calculating HKL is more subject to external effects (Stace 2000). Methodological standardization, especially the degree of chromosomal condensation in the mitotic metaphase, is important for obtaining chromosomal sizes and comparing the results obtained between species (Stace 2000).

Although angiosperms have high diversity in their DNA content, the predominance of a small genome size causes a tendency with modal values equal to 1C = 0.7 pg (Leitch et al. 1998). This distribution, strongly skewed towards small genomes, is associated with the ancestral condition of the group and large genomes could have arose more than once during angiosperm evolution (Leitch et al. 1998; Soltis et al. 2003).

Among the species studied, and in the Solanaceae family in general, we observed a predominance of small genomes (see Bennett and Leitch 2012). The cosmopolitan distribution of the family and occurrence in a wide variety of habitats (D'Arcy 1991; Hunziker 2001; Knapp 2002) are related to some phenotypic characteristics that

are correlated to the low DNA content. Species with low DNA content tend to be found in varying habitats and those with very large genomes appear to be excluded from extreme habitats (Knight and Ackerly 2002).

Despite the predominance of small genomes in Solanaceae, there are some groups with intermediary or large genome sizes, such as species of the genera *Nicotiana*, *Cestrum*, *Capsicum,* and the Cyphomandra clade of *Solanum* (see Bennett and Leitch 2012). There are two main factors associated with increased genome size in plants, polyploidy events or whole genome duplication (Soltis et al. 2003; Leitch and Leitch 2013; Wendel et al. 2015) and an increase in the repetitive elements of DNA (mainly transposable elements) (Leitch and Leitch 2013; Bennetzen and Wang 2014). In Solanaceae, it is likely that the changes in genome size of these groups is related to repetitive elements, since there are few groups with polyploidy relatives causing an increase in DNA content.

CONCLUSIONS

We conclude that some karyotype characters are well conserved in the Solanaceae family at the generic level. Chromosome numbers are very constant, with few reports of polyploidy and aneuploidy and the predominance of chromosome morphology and karyotype symmetry. The family represents a model for karyotypic orthoselection and the karyotype evolution in Solanaceae may have been driven by repetitive DNA reorganization that led to GS diversification, but did not affect chromosome number and morphology.

Acknowledgments: The authors thanks the Comissão de Aperfeiçoamento de Pessoal do Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (Grant number 2016/17096-9) for the financial support. We thank the Empresa Brasileira de Pesquisas Agropecuária (EMBRAPA), unidade Gado de Leite for the infrastructure granted to execute part of the study. We thank Espaço da Escrita – Coordenadoria Geral da Universidade – Universidade Estadual de Campinas (UNICAMP) - for the language services provided.

FUNDING DETAILS

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) under the Grant 2016/17096-9.

REFERENCES

Acosta MC, Bernardello G, Guerra M, Moscone EA. 2005. Karyotype analysis in several South American species of *Solanum* and *Lycianthes rantonnei* (Solanaceae). Taxon 54: 713–723.

Aguilera PM, Debat HJ, García YS, Martí DA, Grabiele M. 2014. *Capsicum rhomboideum*. In: Marhold K, Breitwieser I (eds.), IAPT/IOPB chromosome data 18. Taxon 63: 6, E1.

Bennett MD. 1972. Nuclear DNA content and minimum generation time in herbaceous plants. Proc. Roy. Soc. London, Ser. B, Biol. Sci. 181: 109–135.

Bennett MD. 1998. Plant genome values: How much do we know? Proc Natl Acad Sci USA 95: 2011–2016.

Bennett MD, Leitch IJ. 1995. Nuclear DNA Amounts in Angiosperms. Ann. Bot. (Oxford) 76: 113–176.

Bennett MD, Leitch IJ. 2012. Plant DNA C-values database (release 6.0); [accessed 2018 May 25]. http://data.kew.org/cvalues/

Bennett MD, Smith JB. 1976. Nuclear DNA amounts in angiosperms. Philos. Trans. Roy. Soc. London 274: 227–74.

Bernardello LM, Anderson GJ. 1990. Karyotypic studies in *Solanum* section basarthrum (Solanaceae). Amer. J. Bot. 77: 420–431.

Bernardello LM, Heiser CB Piazzano M. 1994. Karyotypic studies in Solanum Section Lasiocarpa (Solanaceae). Amer. J. Bot. 81: 95–103.

Chase MW, Knapp S, Cox AV, Clarkson JJ, Butsko Y, Joseph J, Savolainen V, Parokonny AS. 2003. Molecular Systematics, GISH and the Origin of Hybrid Taxa in *Nicotiana* (Solanaceae). Ann. Bot. (Oxford) 92: 107–127.

Chiarini F, Barboza G. 2008. Karyological studies in *Jaborosa* (Solanaceae). Bot. J. Linn. Soc.156: 467–478.

Chiarini FE, Bernardello G. 2006. Karyotypic studies in South American species of *Solanum* subgen. *Leptostemonum* (Solanaceae). Pl. Biol. (Stuttgart) 8: 486–493.

Chiarini FE, Santiñaque FF, Urdampilleta JD, Las Peñas ML. 2014. Genome size and karyotype diversity in *Solanum* sect. *Acanthophora* (Solanaceae). Pl. Syst. Evol. 300: 113–125.

Chiarini F, Sazatornil F, Bernadello G. 2018. Data reassessment in a phylogenetic context gives insight into chromosome evolution in the giant genus Solanum (Solanaceae). Syst. Biodivers. 16: 397–416.

Cota JH, Wallace RS. 1995. Karyotypic studies in the genus *Echinocereus* (Cactaceae) and their taxonomic significance. Caryologia 48: 105–122.

Constance L. 1963. Chromosome number and classification in Hydrophillaceae. Britonia 15: 273–285.

D'Arcy WG. 1991. The Solanaceae since 1976, with a review of its biogeography. In: Hawkes JG, Lester RN, Nee M, Estrada, N. eds. Solanaceae III: taxonomy, chemistry, evolution. London: Kew Royal Botanical Gardens; p. 75–137.

Dobigny G, Ducroz J-F, Robinson TJ, Volobouev V. 2004. Cytogenetics and cladistics. Syst. Biol. 53: 470–484.

Dolezel J, Binarová P, Lucretti S. 1989. Analysis of nuclear DNA content in plant cells by flow cytometry. Biol. Pl. 31: 113–120.

Dolezel J, Greilhuber J, Lucretti S, Meister A, Lysák MA, Nardi L, Obermayer R. 1998. Plant genome size estimation by flow cytometry: inter-laboratory comparison. Ann. Bot. (Oxford) 82: 17–26.

Dolezel J, Greilhuber J, Suda J. 2007. Estimation of nuclear DNA content in plants using flow cytometry. Nat. Protoc. 2: 2233–2244.

Ellis RH, Hong TD, Roberts EH. 1985. Handbook of Seed Technology for Genebanks. 2. Compendium of Specific Germination, Information and Test Recommendations. Rome: Handbool for Genebanks 3.

Fernandes TA, Rego LDNA, Nardy M, Yuyama PM, Vanzela ALL. 2009. Karyotype differentiation of four *Cestrum* species (Solanaceae) revealed by fluorescent chromosome banding and FISH. Genet. Mol. Biol. 32: 320–327.

Fregonezi JN, Fernandes T, Torezan JMD, Vieira AOS, Vanzela ALL. 2006. Karyotype differentiation of four *Cestrum* species (Solanaceae) based on the physical mapping of repetitive DNA. Genet. Mol. Biol. 29: 97–104.

García CC, Barfuss MHJ, Sehr EM, Barboza GE, Samuel R, Moscone E, Ehrendorfer F. 2016. Phylogenetic relationships, diversification and expansion of chili peppers (*Capsicum*, Solanaceae). Ann. Bot. (Oxford). 118: 35–51.

Grabiele M, Aguilera PM, Debat HJ, Forni-Martins ER, Martí DA. 2015. *Capsicum baccatum* var. *praetermissum*. In: Marhold K, Breitwieser I eds. IAPT/IOPB chromosome data 18. Taxon 63: 6, E1.

Goodspeed TH. 1954. The genus *Nicotiana*. Waltham (MA): Chronica Botanica Company.

Goldblatt P. 1979. Miscellaneous Chromosome Counts in Angiosperms, II. Including New Family and Generic Records. Ann. Missouri Bot. Gard. 66: 856–861.

Guerra M. 2008. Chromosome numbers in plant cytotaxonomy: Concepts and implications. Cytogenet. Genome Res. 120: 339– 350.

Guerra M. 2012. Cytotaxonomy: The end of childhood. Pl. Biosystems 146: 703–710.

Heiser CB. 1963. Numeración cromosómica de plantas ecuatorianas. Ciencia y Nat. 6:2–6.

Hunziker AT. 2001. Genera Solanacearum: The genera of Solanaceae illustrated, arranged according to a new system. Syst. Bot. Monogr. 29: 221–222.

Knapp S. 2002. Floral diversity and evolution in the Solanaceae. In: Cronk QCB, Bateman RM, Hawkins JA eds. Developmental genetics and plant evolution. London: Taylor and Francis; p. 267–297.

Knapp S, Bohs L, Nee M, Spooner DM. 2004. Solanaceae - A model for linking genomics with biodiversity. Comp. Funct. Genomics 5: 285–91.

Knight CA, Ackerly DD. 2002. Variation in nuclear DNA content across environmental gradients: a quantile regression analysis. Ecol. Lett. 5: 66–76.

Las Peñas M, Chiarini F, Bernardello G, Benítez de Rojas C. 2006. Karyotypes of some species of *Cestrum*, *Sessea*, and *Vestia* (tribe Cestreae, Solanaceae). Caryologia 59: 131–137.

Leitch IJ, Chase MW, Bennett MD. 1998. Phylogenetic analysis of DNA C-values provides evidence for a small ancestral genome size in flowering plants. Ann. Bot. (Oxford) 82: 85–94.

Levan A, Fredga L, Sandberg A. 1964. Nomenclature for centromeric position on chromosomes. Hereditas (Lund) 52: 201–220.

Lima-de-Faria A .1980. Classification of genes, rearrangements and chromosomes according to the chromosome field. Hereditas (Lund) 93: 1–46.

Lujea NC, Chiarini FE. 2017. Differentiation of Nolana and Sclerophylax (Solanaceae) by means of heterochromatin and rDNA patterns. New Zealand J. Bot. 55: 163-177.

Moscone EA. 1992. Estudios de cromosomas meióticos en Solanaceae de Argentina. Darwiniana 31: 261–297.

Marisel S, Chiarini F, Santiñaque FF, Bernadello G, Moscone EA. 2012. Geographical pattern and ploidy levels of the weed *Solanum elaeagnifolium* (Solanaceae) from Argentina. Genet. Resources Crop Evol. 59: 1833–1847.

Melo CAF, Martins MIG, Oliveira MBM, Benko-Iseppon AM, Carvalho R. 2011. Karyotype analysis for diploid and polyploid species of the Solanum L. Pl. Syst. Evol. 293: 227–235.

Mishiba KI, Ando T, MII M, Watanabe H, Kokubun H, Hashimoto G, Marchesis. 2000. Nuclear DNA Content as an Index Character Discriminating Taxa in the Genus *Petunia* sensu Jussieu (Solanaceae). Ann. Bot. (Oxford) 85: 665–673.

Moscone EA. 1993. Estudios cromosómicos en *Capsicum* (Solanaceae) II. Analisis cariotipico de *C. parvifolium* y *C. annum* var. *annum*. Kurtiziana 22: 9–18.

Moscone EA, Lambrou M, Ehrendorfer, F. 1996. Fluores-

cent chromosome banding in the species of *Capsicum* (Solanaceae). Pl. Syst. Evol. 20: 37–63.

Moscone EA. 2003. Analysis of Nuclear DNA Content in *Capsicum* (Solanaceae) by Flow Cytometry and Feulgen Densitometry. Ann. Bot. (Oxford) 92: 21–29.

Moscone EA, Scaldaferro MA, Grabiele M, Cecchini NM, Sanchez García Y, Daviña JR, Ducasse DA, Barboza GE, Ehrendorfer F. 2007. The evolution of chili peppers (Capsicum-Solanaceae): a cytogenetic perspective. Acta Hort. 745: 137–169.

Moyetta NR, Stiefkens LB, Bernadello G. 2013. Karyotypes of South American species of the Morelloid and Dulcamaroid clades (*Solanum*, Solanaceae). Caryologia 66: 333–345.

Olmstead RG, Palmer JD. 1992. A chloroplast DNA phylogeny of the Solanaceae: subfamilial relationships and character evolution. Ann. Missouri Bot. Gard. 79: 346–360.

Olmstead RG, Bohs L, Migid HA, Santiago-Valentin E, Garcia VF, Collier SM. 2008. A molecular phylogeny of the Solanaceae. Molec. Phylogen. Evol. 57: 1159–1181.

Pedrosa A, Gitaí J, Barros e Silva AE, Felix LP, Guerra M. 1999. Citogenética de angiospermas coletadas em Pernambuco: V. Acta Bot. Brasil. 13: 49–60.

Pozzobon MT, Wittmann MT. 2006. A meiotic study of the wild and semi-domesticated Brazilian species of genus *Capsicum* L. (Solanaceae). Cytologia 71: 275–287.

Raven PH. 1975. The bases of angiosperm phylogeny: cytology. Ann. Missouri Bot. Gard. 62: 724–764.

Rego LNAA, da Silva CRM, Torezan JMD, Gaeta ML, Vanzela ALL. 2009. Cytotaxonomical study in Brazilian species of *Solanum*, *Lycianthes* and *Vassobia* (Solanaceae). Pl. Syst. Evol. 279: 93–102.

Romero-da-Cruz MV, Forni-Martins. 2015. *Capsicum chinense.* In: Marhold K., Breitwieser I eds., IAPT/IOPB chromosome data 20 Taxon 64: 6 E34.

Romero-da-Cruz MV, Forni-Martins ER, Urdampilleta JD. 2017. *Capsicum parvifolium.* In: Marhold K., Breitwieser I eds., IAPT/IOPB chromosome data 24 Taxon 66: 1 E16.

Scaldaferro M, Chiarini F, Santiñaque FF, Bernadello G, Moscone EA. 2012. Geographical pattern and ploidy levels of the weed *Solanum elaeagnifolium* (Solanaceae) from Argentina. Genet. Resources Crop Evol. 59: 1833–1847.

Soltis DE, Soltis PS, Leitch IJ, Bennett MD. 2003. Evolution of genome size in the angiosperms. Amer. J. Bot. 90: 1596–1603.

Stebbins GL. 1971. Chromosomal evolution in higher plants. London: Contemporary biology.

Stiefkens L, Bernardello G. 2002. Karyotypic studies in *Lycium* section *Mesocope* (Solanaceae) from South America. Caryologia 55: 199–206.

Stiefkens L, Las Peñas ML, Bernadello G, Levin RA, Miller JS. 2010. Karyotypes and fluorescent chromosome banding patterns in southern African *Lycium* (Solanaceae). Caryologia 63: 50–61.

Sykorová E, Yoong Lim K, Chase MW, Knapp S, Leitch IJ, Leitch AR, Fakjus J. 2003a. The absence of Arabidopsis-type telomeres in *Cestrum* and closely related genera *Vestia* and *Sessea* (Solanaceae): First evidence from eudicots. Plant J 34: 283–291.

Sykorová E, Yoong Lim K, Fakjus J, Leitch AR. 2003*b*. The signature of the Cestrum genome suggests an evolutionary response to the loss of (TTTAGGG)n telomeres. Chromosoma 112: 164–172.

Stace CA. 2000. Cytology and Cytogenetics as a Fundamental Taxonomic Resource for the 20th and 21st Centuries. Taxon 9: 451–477.

Tate JA, Acosta MC, McDill J, Moscone E, Simpson BB, Cocucci AA. 2009. Phylogeny and Character Evolution in *Nierembergia* (Solanaceae): Molecular, Morphological, and Cytogenetic Evidence. Syst. Bot. 34: 198–206.

Urdampilleta JD, Chiarini F, Stiefkens L, Bernardello G. 2015. Chromosomal differentiation of Tribe Cestreae (Solanaceae) by analyses of 18-5.8-26S and 5S rDNA distribution. Pl. Syst. Evol. 301: 1325–1334.

Wu F, Tanksley SD. 2010. Chromosomal evolution in the plant family Solanaceae. BMC Genomics 11: 182.

Zarco CR. 1986. A new method for estimating karyotype asymmetry. Taxon 35: 526–530.

Finito di stampare da
Logo s.r.l. – Borgoricco (PD) – Italia

OPEN ACCESS POLICY

Caryologia provides immediate open access to its content. Our publisher, Firenze University Press at the University of Florence, complies with the Budapest Open Access Initiative definition of Open Access: By "open access" , we mean the free availability on the public internet, the permission for all users to read, download, copy, distribute, print, search, or link to the full text of the articles, crawl them for indexing, pass them as data to software, or use them for any other lawful purpose, without financial, legal, or technical barriers other than those inseparable from gaining access to the internet itself. The only constraint on reproduction and distribution, and the only role for copyright in this domain is to guarantee the original authors with control over the integrity of their work and the right to be properly acknowledged and cited. We support a greater global exchange of knowledge by making the research published in our journal open to the public and reusable under the terms of a Creative Commons Attribution 4.0 International Public License (CC-BY-4.0). Furthermore, we encourage authors to post their pre-publication manuscript in institutional repositories or on their websites prior to and during the submission process and to post the Publisher's final formatted PDF version after publication without embargo. These practices benefit authors with productive exchanges as well as earlier and greater citation of published work.

PUBLICATION FREQUENCY

Papers will be published online as soon as they are accepted, and tagged with a DOI code. The final full bibliographic record for each article (initial-final page) will be released with the hard copies of *Caryologia*. Manuscripts are accepted at any time through the online submission system.

COPYRIGHT NOTICE

Authors who publish with *Caryologia* agree to the following terms:

- Authors retain the copyright and grant the journal right of first publication with the work simultaneously licensed under a Creative Commons Attribution 4.0 International Public License (CC-BY-4.0) that allows others to share the work with an acknowledgment of the work's authorship and initial publication in Caryologia.
- Authors are able to enter into separate, additional contractual arrangements for the non-exclusive distribution of the journal's published version of the work (e.g., post it to an institutional repository or publish it in a book), with an acknowledgment of its initial publication in this journal.
- Authors are permitted and encouraged to post their work online (e.g., in institutional repositories or on their website) prior to and during the submission process, as it can lead to productive exchanges, as well as earlier and greater citation of published work (See The Effect of Open Access).

PUBLICATION FEES

Open access publishing is not without costs. *Caryologia* therefore levies an article-processing charge of € 150.00 for each article accepted for publication, plus VAT or local taxes where applicable.

We routinely waive charges for authors from low-income countries. For other countries, article-processing charge waivers or discounts are granted on a case-by-case basis to authors with insufficient funds. Authors can request a waiver or discount during the submission process.

PUBLICATION ETHICS

Responsibilities of *Caryologia*'s editors, reviewers, and authors concerning publication ethics and publication malpractice are described in *Caryologia*'s Guidelines on Publication Ethics.

CORRECTIONS AND RETRACTIONS

In accordance with the generally accepted standards of scholarly publishing, *Caryologia* does not alter articles after publication: "Articles that have been published should remain extant, exact and unaltered to the maximum extent possible".

In cases of serious errors or (suspected) misconduct *Caryologia* publishes corrections and retractions (expressions of concern).

Corrections

In cases of serious errors that affect or significantly impair the reader's understanding or evaluation of the article, *Caryologia* publishes a correction note that is linked to the published article. The published article will be left unchanged.

Retractions

In accordance with the "Retraction Guidelines" by the Committee on Publication Ethics (COPE) *Caryologia* will retract a published article if:

- there is clear evidence that the findings are unreliable, either as a result of misconduct (e.g. data fabrication) or honest error (e.g. miscalculation)
- the findings have previously been published elsewhere without proper crossreferencing, permission or justification (i.e. cases of redundant publication)
- it turns out to be an act of plagiarism
- it reports unethical research.

An article is retracted by publishing a retraction notice that is linked to or replaces the retracted article. *Caryologia* will make any effort to clearly identify a retracted article as such.

If an investigation is underway that might result in the retraction of an article *Caryologia* may choose to alert readers by publishing an expression of concern.

COMPLYNG WITH ETHICS OF EXPERIMENTATION

Please ensure that all research reported in submitted papers has been conducted in an ethical and responsible manner, and is in full compliance with all relevant codes of experimentation and legislation. All papers which report in vivo experiments or clinical trials on humans or animals must include a written statement in the Methods section. This should explain that all work was conducted with the formal approval of the local human subject or animal care committees (institutional and national), and that clinical trials have been registered as legislation requires. Authors who do not have formal ethics review committees should include a statement that their study follows the principles of the Declaration of Helsinki

ARCHIVING

Caryologia and Firenze University Press are experimenting a National legal deposition and long-term digital preservation service.

ARTICLE PROCESSING CHARGES

All articles published in *Caryologia* are open access and freely available online, immediately upon publication. This is made possible by an article-processing charge (APC) that covers the range of publishing services we provide. This includes provision of online tools for editors and authors, article production and hosting, liaison with abstracting and indexing services, and customer services. The APC, payable when your manuscript is editorially accepted and before publication, is charged to either you, or your funder, institution or employer.

Open access publishing is not without costs. *Caryologia* therefore levies an article-processing charge of € 150.00 for each article accepted for publication, plus VAT or local taxes where applicable.

FREQUENTLY-ASKED QUESTIONS (FAQ)

Who is responsible for making or arranging the payment?

As the corresponding author of the manuscript you are responsible for making or arranging the payment (for instance, via your institution) upon editorial acceptance of the manuscript.

At which stage is the amount I will need to pay fixed?

The APC payable for an article is agreed as part of the manuscript submission process. The agreed charge will not change, regardless of any change to the journal's APC.

When and how do I pay?

Upon editorial acceptance of an article, the corresponding author (you) will be notified that payment is due.

We advise prompt payment as we are unable to publish accepted articles until payment has been received. Payment can be made by Invoice. Payment is due within 30 days of the manuscript receiving editorial acceptance. Receipts are available on request.

No taxes are included in this charge. If you are resident in any European Union country you have to add Value-Added Tax (VAT) at the rate applicable in the respective country. Institutions that are not based in the EU and are paying your fee on your behalf can have the VAT charge recorded under the EU reverse charge method, this means VAT does not need to be added to the invoice. Such institutions are required to supply us with their VAT registration number. If you are resident in Japan you have to add Japanese Consumption Tax (JCT) at the rate set by the Japanese government.

Can charges be waived if I lack funds?

We consider individual waiver requests for articles in Caryologia on a case-by-case basis and they may be granted in cases of lack of funds. To apply for a waiver please request one during the submission process. A decision on the waiver will normally be made within two working days. Requests made during the review process or after acceptance will not be considered.

I am from a low-income country, do I have to pay an APC?

We will provide a waiver or discount if you are based in a country which is classified by the World Bank as a low-income or a lower-middle-income economy with a gross domestic product (GDP) of less than $200bn. Please request this waiver of discount during submission.

What funding sources are available?

Many funding agencies allow the use of grants to cover APCs. An increasing number of funders and agencies strongly encourage open access publication. For more detailed information and to learn about our support service for authors.

APC waivers for substantial critiques of articles published in OA journals

Where authors are submitting a manuscript that represents a substantial critique of an article previously published in the same fully open access journal, they may apply for a waiver of the article processing charge (APC).

In order to apply for an APC waiver on these grounds, please contact the journal editorial team at the point of submission. Requests will not be considered until a manuscript has been submitted, and will be awarded at the discretion of the editor. Contact details for the journal editorial offices may be found on the journal website.

What is your APC refund policy?

Firenze University Press will refund an article processing charge (APC) if an error on our part has resulted in a failure to publish an article under the open access terms selected by the authors. This may include the failure to make an article openly available on the journal platform, or publication of an article under a different Creative Commons licence from that selected by the author(s). A refund will only be offered if these errors have not been corrected within 30 days of publication.

2019
Vol. 72 – n. 3

Caryologia

International Journal of Cytology, Cytosystematics and Cytogenetics

Table of contents

€ 25,00