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Chromosomal Studies of Polyploidization in Pisum SativumL. Induced by Colchicine

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دراسات كروموسومية حول تعدد الصيغ الصبغية المستحث بالكولشيسين في نبات البازلاء (*Pisum sativum* L.*)

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Abstract:
Polyploidy is an important element in flowering plant evolution and ecology. Understanding the type and degree of morphological differentiation among ploidy levels within a species may aid in better understanding how polyploidy affects biodiversity. Polyploidy can occur naturally as unreduced gametes develop, or it can be caused artificially by doubling the number of chromosomes in somatic cells. Polyploidy is induced synthetically with the aid of mitotic inhibitors when natural polyploid plants are unavailable. Colchicine is a widely used mitotic inhibitor for the induction of polyploidy in plants during their cell division by inhibiting the chromosome segregation.
In order to explore the possibilities of producing polyploidy Pisum sativum.L., the diploid seedlings of this variety was treated with four concentrations of aqueous colchicine (0.1, 0.2, and 0.3%) using soaking method for 9 and 18 hrs duration times. For detection of induced polyploidy, a wide array of chromosomal consequences and DNA were studied and investigated for up to 4 plants at each treatmenFollowing colchicine treatment, changes occur in the MI; there is a significant increase 18 h after treatment with 0. 3 and 0.2 colchicine to reach 45.8% and 25.4%, respectively, and the differences were highly significant compared to the control values 7. 8%.

Keywords: Pisum sativumL, colchicine, mitotic indexEach

الملخص
يُعد تعدد الصيغ الصبغية عنصرًا مهمًا في تطور النباتات المزهرة وبيئتها. قد يساعد فهم نوع ودرجة التمايز المورفولوجي بين مستويات الصيغة الصبغية داخل النوع الواحد في فهم أفضل لكيفية تأثير تعدد الصيغ الصبغية على التنوع البيولوجي. يمكن أن يحدث تعدد الصيغ الصبغية بشكل طبيعي أثناء نمو الأمشاج غير المختزلة، أو يمكن أن يحدث بشكل اصطناعي عن طريق aمضاعفة عدد الكروموسومات في الخلايا الجسدية. يتم تحفيز تعدد الصيغ الصبغية صناعيًا بمساعدة مثبطات الانقسام عندما لا تتوفر نباتات متعددة الصيغ الصبغية طبيعية. يُعد الكولشيسين مثبطًا للانقسام يستخدم على نطاق واسع لتحفيز تعدد الصيغ الصبغية في النباتات أثناء انقسام خلاياها عن طريق تثبيط انفصال الكروموسومات
لاستكشاف إمكانيات إنتاج تعدد الصيغ الصبغية في نبات البازلاء***(Pisum sativum L.)*** ، عولجت الشتلات ثنائية الصيغة الصبغية من هذا الصنف بأربعة تراكيز من الكولشيسين المائي (0.1، 0.2، و0.3%) باستخدام طريقة النقع لمدة 9 و18

ساعة. وللكشف عن تعدد الصيغ الصبغية المستحث، دُرست مجموعة واسعة من التغيرات الكروموسومية والحمض النووي (DNA)وفُحصت لما يصل إلى 4 نباتات في كل معالجة. بعد المعالجة بالكولشيسين، تحدث تغيرات في مؤشر الانقسام الخلوي(MI) ؛ حيث لوحظت زيادة ملحوظة بعد 18 ساعة من المعالجة بتركيزي 0.3 و0.2 من الكولشيسين لتصل إلى 45.8%و25.4% على التوالي، وكانت الاختلافات ذات دلالة إحصائية عالية مقارنة بقيم التحكم البالغة 7.8

الكلمات المفتاحية: Pisum sativumL ، الكولشسين، مؤشر الانقسام المتساوي

Introduction

polyploidy is an important genomic feature for all plants, especially in plants where most or all angiosperms are polyploids or have polyploidy origins (Ainouche and Jenczewski, 2010). Polyploidy (or whole-genome duplication) is a widespread feature of plant genomes, but its importance to evolution has long been debated due to the inefficiency of selection when genes are masked by various copies, polyploids have been postulated to be evolutionary dead ends (Mayrose et al., 2011). Almost 70% of all angiosperms, naturally, are polyploids (Nasirvand et al., 2018). Generally speaking, polyploids may differ from their progenitors in morphological, ecological, physiological, and cytological characteristics (Ramsey and Schemske, 2002). Results obtained show that the polyploidy is one of the successful methods in the improvement of some plants such as apples. Polyploidy can also be induced through the use of chemical doubling agent's colchicine (Tiwari Besides genetic approaches like polyploidization, other treatments can also improve plant growth. For example, treating plants with natural biostimulants like licorice and yeast extracts significantly increased vegetative growth in faba bean (Adam et al., 2022).

Pea (Pisum sativum L.) was the original model organism used in Mendel's discovery (1866) of the laws of inheritance, making it the foundation of modern plant genetics) .Economically, legumes represent the second most important family of crop plants after Poaceae (grass family) (Smýkal et al., 2012). Recent cytological and genetic research has proved that all species have a chromosome number of 2n = 14, and no natural polyploid forms have been discovered (Jaranowski and Micke, 1985) g peas, have long been important components of the human diet due to their content of starch, protein and other nutrients. These health benefits derive mainly from the concentration and properties of starch, protein, fibre, vitamins, minerals and phytochemicals in peas (Dahl et al., 2012).

Polyploidy can change morphological, phonological, physiological and ecological character of plant to form and incidence of polyploidy

Several chemicals (oryzalin and colchicine) have been used for doubling the chromosome number of ornamental plants; however, colchicine, owing to its long track record, is the most commonly used chemical agent for polyploidy (Ye et al., 2010) and (Allafe et al.,2024) .colchicine, an alkaloid antimitotic agent obtained from seeds of Colchicum autumnale, belongs to family Lilliaceae and has been used for chromosome doubling to induce experimental polyploids (Kazi, 2012). Colchicine is applied at very low concentrations because high concentrations are highly toxic to plant cells (Caperta et al., 2006). Colchicine interferes with the cytoskeleton by inducing microtubule depolymerization, which prevents the formation of mitotic spindle fibers. As a result, chromatids fail to localize at the cellular poles during metaphase and eventually become enclosed, together, in a new nuclear membrane and the cell proceeds to interphase with twice as many chromosomes (Caperta et al., 2006).

Recent physiological research indicates that plant growth regulators are among the key factors governing essential biological processes related to seed germination and early vegetative development. Gibberellic acid (GA_3) is recognized as one of the most influential regulators due to its pronounced role in activating enzymes responsible for mobilizing stored nutritional reserves within seeds, as well as in promoting cell division and elongation. Previous studies have demonstrated that pre-sowing seed treatment with gibberellic acid significantly enhances germination quality parameters, seedling vigor, and the development of both root and shoot systems. These findings underscore the importance of integrating hormonal regulation with chemical treatments to improve overall plant physiological performance (Allafe & Adam, 2022) and(Allafe et al.,2021).

Material and methods

This study was conducted at university laboratories of the tobruk university_libya faculaty of science botany department in 2020-2021.

seeds *pisum sativum l* .plant were provided from local market

Seed surface sterilization:

Prior to plating, seeds were surface sterilized with 70 % (v/v) for 5 minutes followed by 1 minute incubation in 100 % (v/v). Afterward, the seeds left to dry on sterile filter paper in sterilize laboratory work cabinet . .

Treatment of *V. peas* L. with colchicine:

Ten 50 ml centrifuge tubes were used, each one containing 20 disinfected seeds. Thereafter, the seeds were

immersed for 9 and 18 h at room temperature in control different concentration of colchicine (0.0%, 0.1%, 0.2%, 0.3%) for 9 and 18 hours interval. Aqueous solutions of colchicine dissolved in distil water. After each treatment, the seeds were washed under running tap water for five times, with each wash step lasting 5 minutes.

Chromosomal analysis:

For mitotic index, soaked seeds spread over moist cotton and kept in Petri-dishes (15 cm diameter) at 25 ± 2 °C for further observation. The secondary root tips (FIGURE 2) of germinated seeds were used as a source of mitotic cells. The selected root tips were washed in water after treated with selected colchicine concentrations , fixed in a solution of glacial acetic acid and absolute alcohol in the ratio of 1:3 for 24 h and stored in 70% alcohol until further use. The fixed roots were washed and hydrolyzed at 60 °C for 10 min, in 1 N HCl. Root tips were stained with 2% acetocarmine (Measure out 55 ml H2O into a 100-ml cylinder and pour into a 300-ml beaker containing a stir bar. Measure out 45 ml of glacial acetic acid using a pipette and add to beaker containing H2O. Weigh out 2. 0 grams of carmine and add to the 45% acid solution. Place a beaker on a stir plate in a fume hood. Boil gently for 5 minutes on highest setting, become dark red then filtered in dark bottle placed in refergitor) and squashed on a slide, mounted and observed under microscope. The cells were scored under the microscope for different types of cell mitotic figure were made for each of the treatment and control. Effect of colchicine treatment and control on different chromosome plates were observed under light microscope (BX45; Olympus Optical Co. Ltd.) at 400 × To determine the effects of polyploidy on cell size, chromosome number and mitotic index, by scoring 1,000 cells from each of 4 slides for each treatment , giving a total of 4,000 cells. were scored in control group and in each treated group. The mitotic index MI were calculated for both treated and untreated samples using the formula(Grant, 1982).

$$\text{Mitotic index(MI)} = \frac{\text{number of cell in mitosis(P+M+A+T)} * 100}{\text{Total mumber of cell}}$$

Results and discussion

effect on mitotic index

The rate of cell division expressed by mitotic index (MI) shows a general TABEL 3 enhance effect on mitotic index values as the concentrations increased and the time of treatments prolonged, results in an increase in the MI over the control value. Mitotic indices of the controls and the treated roots have been compared, control shows a normal mitotic distribution represented in FIGUR 13 and the difference between these mitotic indices is highly significant. These differences were not significant in control and T1 but were significant in T2 and T3 FIGURE 4 In general, the Most of the actively dividing cells were in prophase and few in metaphase, anaphase and telophase stages of cell division

Table (1): Number, percentage and phase index of the different stages of mitosis and interphase for the control and treated cells at different concentrations of colchicine after 9 and 18 hours treatments. TDC: total dividing cells, MI: mitotic index PPI: Prophase index, MPI: Metaphase index, API: Anaphase index, TPI: Telophase index.

	Cell division stages							Phases Index(PhI)			
Conc	Pro	Meta	Ana	Telo	Total	TDC	MI	PPI	MPI	API	TPI
Con	160	31	29	60	3610	280	7.8	56.4%	11.06	10.4	22.2
T1 9H	527	8	8	44	3870	587	15.1	87.9%	1.7	1.6	8.84
T2 9H	606	1	8	16	3830	631	16.5	96.3%	0.15	1.34	2.25
T3 9H	831	6	0	47	3640	884	24.5	16.5%	0.7	0	5.3
Con	160	31	29	60	3610	280	7.8	56.4%	11.06	10.4	22.2
T1 18H	700	2	0	55	3680	757	20.6	92.2%	0.003	0	7.4
T2 18H	739	19	6	118	3480	882	25.4	83.3%	2.2	0.64	13.8
T3 18H	1752	0	0	21	3880	1773	45.8	99%	0	0	1.7

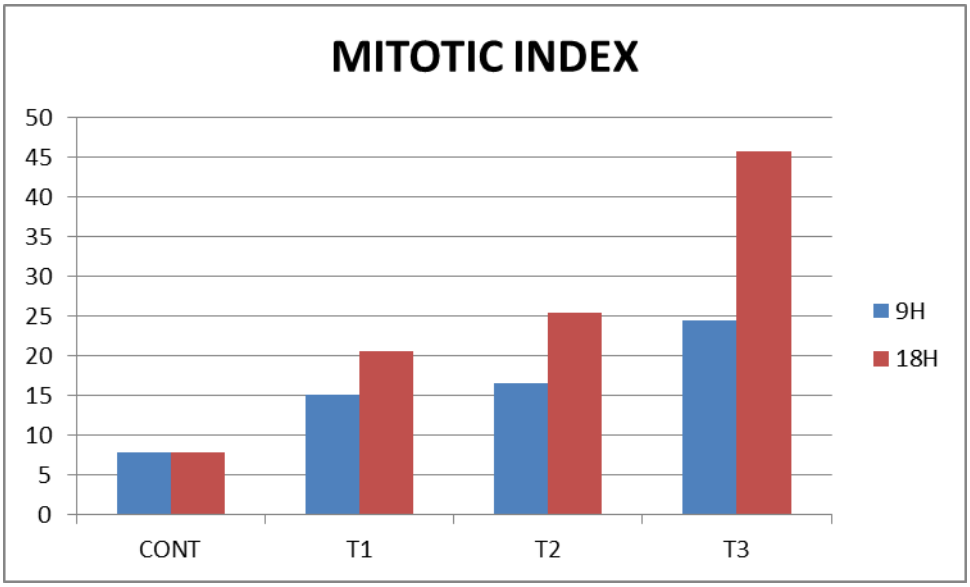


FIGURE 1 Mitotic index in (Pisum sativum L.). root tips treated with increasing colchicine concentrations at 9 and 18 hours

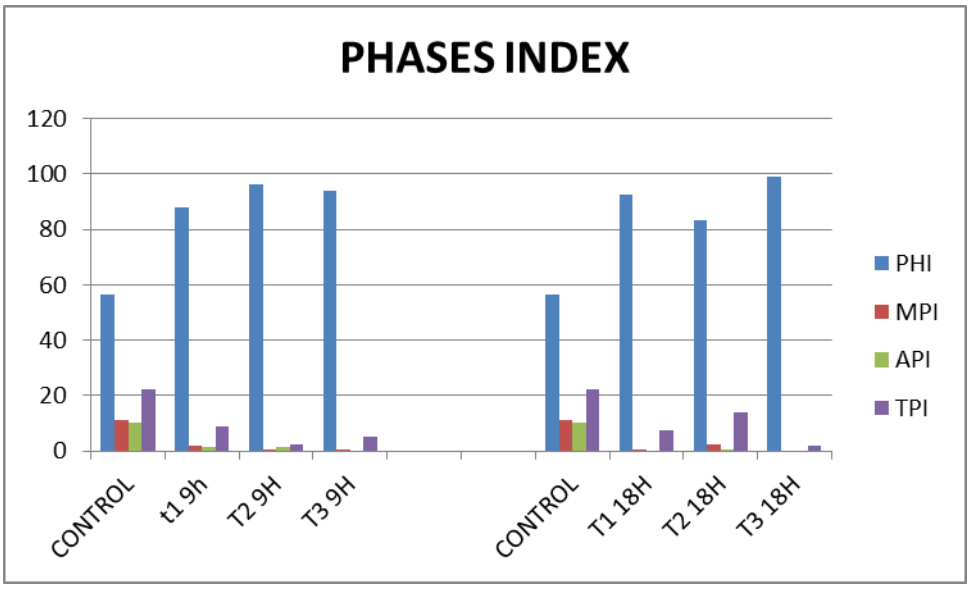


FIGURE 2 phases index in (Pisum sativum L.). root tips treated with increasing colchicine concentrations at 9 and 18 hours

It can be seen in FIGURE 1 that the MI was altered significantly by of the most of used treatments, the values for MI after treatment ranged from .7.8%to 45.8% compared with the control value of 7.8%. Surprisingly, the highest MI value in treated groups was 45.8% after 18 hrs treated with the T3 concentration of colchicines (FIGURE. 1).

Disccution:

Colchicine for polyploidy induction

The success of the polyploidization process depends on numerous factors, such as the type of explant, the anti-mitotic agent used and its ability to penetrate the cells, the concentration applied and the duration of the exposure (Ascough *et al*., 2008)

The use of seeds and seedlings as explants are also particularly suitable for most plant species. Colchicine is an important mutagen

that works by preventing the microtubules formation and doubles the number of chromosomes. It is commonly used to develop polyploid plants and functions as a mitotic poison by producing many mutagenic effects on plants (El-Nashar and Ammar, 2016). As microtubules function in chromosome segregation, colchicine induces polyploidy by preventing the segregation of chromosomes during meiosis that results into half of the gametes containing double the chromosome number than usual. Whereas, half of the gametes do not contain any chromosomes and produce embryos with doubled chromosome numbers (Ade and Rai, 2010). Colchicine not only

helps in the doubling of chromosomes, but it also induces mutation in plants. Plants that have been mutated through colchicine are known as colchicine-mutants.

For this reasons, colchicine is a highly potent antimitotic agent which has been successfully used for inducing polyploidy in many plant species (Trojak-Goluch and Skomra, 2013). As result, induction variation through chromosome doubling is dependent on a balance of colchicine concentration and exposure time that doubles the genomic content of each cell and effect the cell division interfering with the cellular mechanism (Eng and Ho, 2019 ; Ascough *et al*., 2008) The results of this study indicate that the effect of colchicine on plant cells is not limited to chromosomal alterations alone, but also extends to morphological and physiological changes that are directly influenced by the concentration of the substance and the duration of tissue exposure. It was observed that moderate concentrations of colchicine enhance several vegetative traits of the plant, such as increased stem length, number of leaves, and biomass. In contrast, higher concentrations lead to a marked decline in growth indicators due to cellular toxicity. These findings are consistent with what was reported by (Allafe & Adam, 2022) and The relationship between colchicine and the mitotic index

Colchicine in current experiment, however, also has effects on mitosis which normally require a longer period of treatment: these are;

i. an increase in M I
ii. an increase in the numbers of cells in prophase and metaphase
iii. a change in the prophase: metaphase ratio
iv. polyploid cells come into division.

In the comparative study, the concentration treatments of colchicine led to an increase in the number of lateral roots, which is an indicator of cell division reorganization as a result of microtubule disruption. This is confirmed by the large increase in prophase and chromosome duplication observed in the results of this research.(Allafe and Elmosmary,2022)

In the experiments described in this section, the MI sharply increased at the end of a 18 hour treatment (FIGURE 4). An increase in the MI has been reported frequently (Eigsti and Dustin, 1955)and is due to the accumulation of cells in metaphase. The increase in MI within a 9 hour period may or may not be significant depending on the concentration of colchicine used, but it is highly significant after a 18 hour recovery period I. e. MI continues to show an increase even 18 hours after the end of treatment. (Duhoky and Zibra, 2019)

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