J. Agr. Sci. Tech. (2014) Vol. 16: 1683-1698

Observations on the Cytology and Karyogram of an *Onobrychis viciifolia* Scop. New Variety in Callus, *In vivo* and *In vitro* Cultures

S. Mohajer[1*], and R. M. Taha[1]

ABSTRACT

Genus *Onobrychis* is an important forage crop consisting of approximately 130 annual and perennial species. The present research focused on the cytological studies of Embryogenic (E) and Non-Embryogenic (NE) callus as well as root meristem cells of *in vitro* and *in vivo* grown plants. On the contrary to the previous studies, a diploid *viciifolia* variety with 2n= 2x= 16 chromosomes was observed for the first time in *Onobrychis viciifolia* Scop Syn. *Onobrychis sativa* L. Mitotic division was in general regular for all the growth conditions, but some such division irregularities as cytomixis, binucleate cells, micronucleus, asynchronous nucleus and chromosome bridge were also observed particularly in non-embryogenic callus samples. Ratio of nuclear to cell area of callus was slightly less than those in *in vivo* and *in vitro* values. Polyploidy level of DNA (C-value) was only indicated in callus. G_1 had the highest percentage of nucleus among different stages of the interphase in *in vitro* and *in vivo* growth cultures. Karyotypic formula was 10 m+6 sm and a secondary constriction was distinguished in the short arm of the second pair chromosomes. Ultimately, scanning electron microscopy was employed to differentiate between root cells of the *in vitro* and *in vivo* growth systems.

Keywords: Callus, Mitotic, Karyotype, *Onobrychis viciifolia,* Polyploidy.

INTRODUCTION

Description of the regenerative behaviour of somatic cells is an important prerequisite to reveal the ways, in which cell proliferation is regulated, and how the non-organized cells, i.e., callus, can be stimulated to embryonic organization. The different behaviours of Embryogenic (E) and Non-Embryogenic (NE) callus can also be detected through noticeable differences within their cells. Cytological analyses are usually performed to assess the mitotic process in experimental varieties and hybrids. Hybridization of the close genetic species produces regular chromosome hybrids, while the offspring of those more distantly related species has meiotic irregularities (Marfil *et al.,* 2006). High irregular cell division and polyploidy levels have been already indicated by researchers for different plant species (Nirmala and Kaul, 1993; Miller and Venable, 2000; Stace, 2000). Although some *in vitro* cytological studies of *Onobrychis viciifolia* have been mentioned regarding karyological parameters (Mesicek and Sojak, 1992) still no research has been conducted on mitotic division of the callus cells in this species.

It is difficult to recognize the taxonomy classification of the *Onobrychis* genus due to several attitudes about this species delimitation (Duman and Vural, 1990; Aktoklu, 2001). Unlike the morphological characteristics of plants that can be re-valued by botanists, results of both karyological analysis and mitotic behaviour are referred to information references for many researchers.

[1] Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia.
[*] Corresponding author; e-mail: mohajer.ae@gmail.com

[DOR: 20.1001.1.16807073.2014.16.7.6.2]

Onobrychis species were found to be consisted of three polyploidy levels of (2n= 2x= 14, 2n= 4x= 28, 2n= 8x= 56 and 2n= 2x= 16, 2n= 4x= 32) and two basic chromosome numbers of (x= 7 or x= 8) (Abou-El-Enain, 2002). Diploid level of *O. viciifolia* is not much known. Most of the previous studies have only focused on tetraploid level with 2n= 4x= 28 chromosomes in the species (Kidambi *et al.,* 1990b). Tetraploid strain was observed in 20 different sainfoin species using polidy analysis technique by Negri *et al.* (1987). Similar result was indicated by Tamas (2006), whilst the mean chromosome length was 3.39µm in general.

Researchers have succeeded in deciphering an unknown mechanism that plays a key role in cell shape changes during mitosis (Werner *et al.,* 2013). They investigated the transient degradation of a protein that regulates specific structures of the mechanical scaffold of the cell, the actin cytoskeleton. In the same manner, scanning electron microscopy was employed to observe and compare root cells of the *in vitro* and *in vivo* growth systems throughout the present study.

The present work aimed at increasing the knowledge of the mitotic division at diploid level, to recommend a new variety of *Onobrychis viciifolia* and compare the *in vitro*, *in vivo* and callus cytological studies of cells based on the DNA *C*-values, Mitotic Index (MI), cell and nuclear areas, ratio of nuclear to cell areas and ultra-structure of root cells. A unique staining protocol and some mitotic abnormalities were also evaluated.

MATERIALS AND METHODS

Plant Materials

The study was carried out at the Institute of Biological Sciences, University of Malaya, Kuala Lumpur, Malaysia. Seeds of *Onobrychis viciifolia* were obtained from the natural-agricultural resources of Iran situated at 34° 64' North and 50° 78' E longitude with an elevation of 930 meters above mean sea level.

One hundred seeds of sainfoin were germinated on moist cotton wool in Petri dishes and MS medium for 2 weeks to obtain a standard growth curve for the primary roots. The primary root length of the population was measured once daily at a fixed time and the mean root length of each sample recorded and plotted against time.

For *in vitro* cytological studies, sterilized seeds were cultured again on full strength MS medium. Root tips were excised after one week for slide preparation, when the secondary roots were appearing. For *in vivo* study, the seeds were cultured in black soil: red soil ratio of 1:1 and transferred to a growth chamber and maintained at 24±2°C (16 hours photoperiod and 8 hours dark period).

Embryogenic and Non-embryogenic Callus Production

After 3-4 weeks of seed incubation on MS media, stem explants of sainfoin were cut into small pieces (2-3 mm) from aseptic seedlings using fine sterile forceps of a sharp sterile blade. For the callus induce, the explants were inoculated on MS medium fortified with different concentrations of BAP, NAA and BAP, IBA (Mohajer *et al.,* 2012). The explants were maintained for 3 weeks in growth room at 24±2°C, 70% humidity and 16 hours light photoperiod provided by cold fluorescent lamps. One week past of last subculture, samples were collected. To ensure that the NE callus had truly lost regeneration capacity and did not contain the E cells, double staining method (Gupta *et al.,* 1987) was employed. After 3 weeks of culture, double staining technique was employed to distinguish embryogenic cells from non-embryogenic ones. Two step procedure (Preparation of 2% Acetocarmine and 0.5% Evan's Blue) was followed for the method. Two (2) g of carmine was weighed out and added to acid solution of 45% (Mohajer *et al.,* 2012). Embryogenic calluses was observed with red nuclei in double staining method, while non-

[DOR: 20.1001.1.16807073.2014.16.7.6.2]

embryogenic cells were indicated blue nuclei.

Slide Preparation Methods and Image Analysis

Root tip meristems obtained from seedlings were pre-treated in 8-hydroxyl-quinalin (2 mM) at 4°C for 5 hours, fixed in Farmer`s fluid (3 ethanol: 1 acetic acid) and Carnoy`s solution (1 glacial acetic: 3 chloroform: 6 ethanol) in three different durations of 15 and 60 minutes as well as overnight. The samples were hydrolyzed in 5N HCL for 40 minutes at room temperature (cold method) and 1N HCl for 10 and 30 minutes at 60ºC (hot method). Following washing in distilled water, root tips were placed in Feulgen`s reagent for 3 hours. Root cells were also treated by squashed technique and stained with 2% acetocarmine. Subsequently, roots were squashed on slides and immersed with 45% (v/v) acetic acid. Cover slides were then mounted on the slides with DPX (Di-N-Butyle Phthalate in Xylene).

Cytometric Parameters

Permanent slides of *in vitro*, *in vivo* and callus were analyzed for such cellular behaviour parameters, as nuclear and cell areas, mitotic index as well as chromosome count. The experiments were carried out using a light microscope (Zeiss Axioscope, Germany) connected to a Sony Video Camera. Image analyzer was employed for measurement of mean cell as well as nuclear areas. The *2C* value was determined by calibrating with the Integrated Optical Density (IOD) of reference nuclei. A total of 150 interphase nuclei from each sample were scored. The gray OD of nuclei in different cell cycle phases were estimated according to the *C* values of the cells, i.e., 0-2.2 *C* (G_1), 2.2-3.6 *C* (S-phase), 3.6-4.8 *C* (G_2) and > 4.8 *C* was determined for ploidy histogram (Evans and Van`t Hof, 1974).

Karyotype Analysis

The following parameters were estimated at the end of prophase step to characterize the karyotype numerically: Long Arm (LA), Short Arm (SA), Total Length *(TL= LA+SA)*, Relative Length Percentage *[RL %=(TL/ ΣTL)×100]*, Value of Relative Chromatin *(VRC= ΣTL/n)*, Arm Ratio *(AR= LA/SA)* and Centromeric Index *[CI= SA/(LA+SA)]* (Hesamzadeh and Ziaie, 2009). Karyotype asymmetry was estimated by different methods namely: the Total Form percentage *[TF%= (ΣSA/ΣTL)×100]* by Huziwara (1962); As *K%= [(ΣLA/ΣTL)×100]* by Arano (1963), Difference of Relative Length *(DRL%= MaxRL%–MinRL%)* by Romero Zarco (1986) and relative length of shortest chromosome (S%). Karyotypic evolution was also determined utilizing the Symmetry Classes of Stebbins (SC) (Stebbins, 1971). Classification of karyotype symmetry is based on the ratio of the smallest to the longest chromosome within the complement. If the ratio is less than 2:1, chromosome set is termed *A*. If the ratio situates between 2:1 and 4:1, chromosome set belong to *B* group; and further within class categorization is indicated by numerical prefixes 1, 2, 3... in increasing order of proportion of chromosomes with arm ratio < 2:1 on 0–1 scale (0–0= 1, 0.01–0.5= 2, 0.51–0.99= 3, 1.0= 4). Karyotypic formula was determined by chromosome morphology based on centromere position and *r*-value in classification of Levan *et al.* (1964).

Romero Zarco (1986) provided an alternative method for an estimation of the karyotypic asymmetry by using quantification and graphic representation. He proposed two numerical parameters to estimate karyotypic asymmetry. The first one was named the intrachromosomal asymmetry index (A1) and the second one was termed the interchromosomal asymmetry index (A2).

Interachromosomal asymmetry index *(A_1)= 1-[Σ(SA/LA)/n],* where SA and LA are the mean lengths of short and long arms of each pair of homologous, respectively and *n* is the number of homologous, Interchromosomal

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asymmetry index (A_2)= s/x, where s and x are the average of standard deviation and mean of chromosome length, respectively. percentage of Symmetry Index [SI%= (Length of smallest chromosome/Length of longest chromosome)×100]; Centromeric Gradient value [*CG*%= (Length of median short arm/Length of median chromosome)×100]; Dispersion Index (*DI*%= A_2×*CG*) (Lavania and Srivastava, 1992), degree of asymmetry of karyotype $A=\sum_{i=1}^{n}[(LA-SA)/TL]/n$ by Watanabe *et al.* (1999).

Two indices (*Syi* and *Rec*) were also used for the analysis of karyotypic asymmetry and the index of chromosomal size similarity. *Syi* and *Rec* indices were evaluated through the formulas (Greilhuber and Speta, 1976):

Syi%= [(Mean length of the short arms/Mean length of the long arms)×100]

$Rec=[(\sum_{i=1}^{n}(CLi/LC)/n) \times 100]$ where, *CLi* is the length of each chromosome to the longest one (LC).

Scanning Electron Microscopy (SEM)

Root specimens of both *in vitro* and *in vivo* growth cultures were treated with the following solutions of: 1:1 (v/v) glutaraldehyde (4%), and subsequently phosphate buffer solution at room temperature for 1 h, phosphate buffer solution and distilled water in 1:1 mixture for 30 minutes, then osmium tetroxide (4%) at 48ºC for 14 hours. After rinsing the samples with distilled water, the tissues were immersed in an ethyl alcohol series (10–100%) within 15 minutes intervals followed by; (1) 3:1 ethyl alcohol and acetone for 20 minutes, (2) 1:1 ethyl alcohol and acetone for 20 minutes, (3) 1:3 ethyl alcohol and acetone for 20 minutes, and (4) 100% acetone for 20 minutes. The final step was repeated four times. The replacement of acetone with carbon dioxide was carried out several times using a critical point dryer. Eventually, the samples were coated with gold powder for 1 minute, before observation through SEM (JEOL 6400).

RESULTS

Seed germination and root lengths were determined under *in vitro* and *in vivo* growth cultures. Both cultures' seeds started germinating their secondary roots after one week's time, while *in vivo* indicated the longer root length during the first week (Figure 1). Legume crops like sainfoin usually have

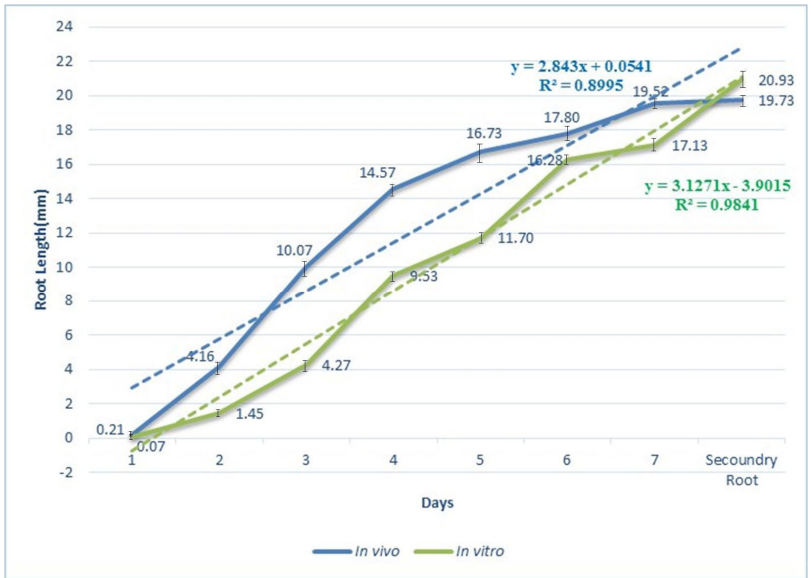

Figure 1. Standard graph of root length in *in vitro* and *in vivo* growth cultures before secondary root.

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Table 1. Comparison of different staining methods for chromosome observation of mature root tissues.[a]

Fixation	Time (Min)	Hydrolysis	Time (Min)	Stain	Clarification	Fixation	Time (Min)	Hydrolysis	Time (Min)	Stain	Clarification
Farmer	15	Hot	10	Carmine	5cde	Carnoys	60	Hot	30	Feulgen	5cde
Farmer	15	Hot	10	Feulgen	3b	Carnoys	Overnight	Hot	10	Carmine	5cde
Farmer	15	Hot	30	Carmine	4.5cd	Carnoys	Overnight	Hot	10	Feulgen	5cde
Farmer	15	Hot	30	Feulgen	4.5cd	Carnoys	Overnight	Hot	30	Carmine	5cde
Farmer	60	Hot	10	Carmine	4c	Carnoys	Overnight	Hot	30	Feulgen	1.5a
Farmer	60	Hot	10	Feulgen	1.75a	Carnoys	60	Hot	30	Carmine	3.5bc
Farmer	60	Hot	30	Carmine	2.5ab	Farmer	15	Cold	40	Carmine	5cde
Farmer	60	Hot	30	Feulgen	1.75a	Farmer	15	Cold	40	Feulgen	4.5cd
Farmer	Overnight	Hot	10	Carmine	5cde	Farmer	60	Cold	40	Carmine	4c
Farmer	Overnight	Hot	10	Feulgen	4.5cd	Farmer	60	Cold	40	Feulgen	4.5cd
Farmer	Overnight	Hot	30	Carmine	5cde	Farmer	Overnight	Cold	40	Carmine	5cde
Farmer	Overnight	Hot	30	Feulgen	4c	Farmer	Overnight	Cold	40	Feulgen	4c
Carnoys	15	Hot	10	Carmine	4c	Carnoys	15	Cold	40	Carmine	5cde
Carnoys	15	Hot	10	Feulgen	3.5bc	Carnoys	15	Cold	40	Feulgen	3.5bc
Carnoys	15	Hot	30	Carmine	4c	Carnoys	60	Cold	40	Carmine	5cde
Carnoys	15	Hot	30	Feulgen	2ab	Carnoys	60	Cold	40	Feulgen	4c
Carnoys	60	Hot	10	Carmine	3.5bc	Carnoys	Overnight	Cold	40	Carmine	5cde
Carnoys	60	Hot	10	Feulgen	5cde	Carnoys	Overnight	Cold	40	Feulgen	2.5ab

[a] The means of the populations with same small letters were not significantly different as per Duncan's multi-range test at $P< 0.05$.

very small chromosomes leading to a complex cytological study for observation and analysis. Since there was no precise protocol for staining and slide preparation in sainfoin, various manners of fixation, hydrolysis and stain materials were carried out on mature root tissues. Clarity of cells was scored based on the percentage of the cell's chromosomes (1= 100%; 5= 0%). Finally, Carnoy`s solution (24 hours) with hot hydrolysis (30 minutes) and Feulgen`s reagent was chosen to evaluate the cytological study (Table 1). Either more than 50% of the nuclei were in G_1 stage of the interphase, or no significant difference observed between *in vitro* and *in vivo* growth cultures at G_1 and *S* stages. Embryogenic and non-embryogenic callus had the highest and the lowest nuclei sample percentages in the *S* and G_2 stages, respectively. In callus tissues, two categories of cell cycling were distinguished; the first one had *2C* value of DNA content, while the second one had value more than *2C* (Polyploidy) (Table 2). Based on the DNA (C-value) distribution in the interphase, the values were less than 4 for both *in vitro* and *in vivo* growth cultures, which confirmed diploid cells. In addition, non-embryogenic callus showed the higher variation of the polyploidy levels in comparison with E callus (Figure 2). Mitotic index as a parameter of cell activity and proliferation increased from 20.56% in the E callus to 22.41% in the NE cells. More than 70% of nuclei were observed in the interphase stage of the mitotic cycle in *in vivo*, *in vitro* and callus tissues. Low percentages of the

Table 2. Percentage of the nuclei in interphase and polyploidy in different tissues.

	Cell cycle phase (%)			
Cell line	G_1 [a]	S [b]	G_2 [c]	Polyploidy (%)
In vitro	55.31^{a}±0.32	34.04^{b}±0.15	10.63^{b}±0.11	-
In vivo	59.25^{a}±0.24	33.31^{b}±0.27	7.40^{c}±0.06	-
E.[d] callus	19.67^{c}±0.17	37.93^{a}±0.25	13.35^{a}±0.14	29.03^{b}±0.15
Non-E.callus	32.25^{b}±0.22	29.03^{bc}±0.13	6.45^{c}±0.09	32.25^{a}±0.24

[a] Gap1, [b] Synthesis, [c] Gap2, [d] Embryogenic.

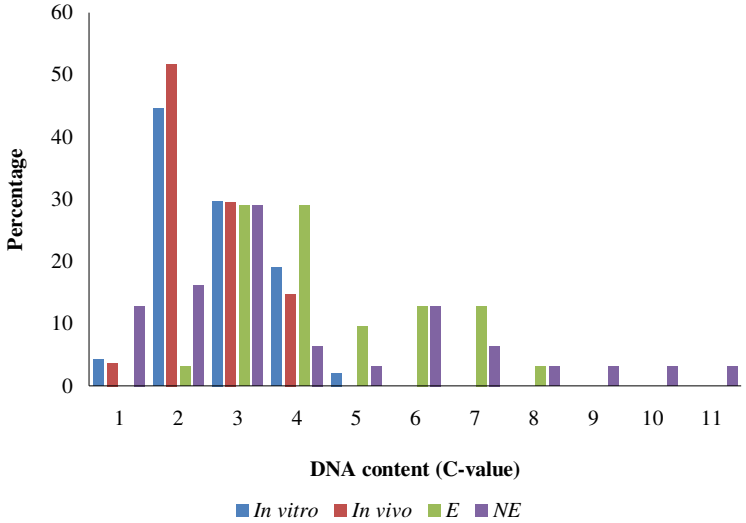

Figure 2. Distribution of DNA (C-value) of interphase cells in various tissues of *Onobrychis viciifolia.* E: Embryogenic callus, NE: Non- Embryogenic callus.

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anaphase and telophase showed that cells spent a short period of time in division cycle within these two stages (Table 3). Mean nuclear and cell areas of the NE callus amounted to 89.578 and 619.802 μm^2, which indicated less than those of E callus (125.021 and 745.327 μm^2, respectively). Either ratio of nuclear to cell area was higher in *in vitro* growth culture than intact sample, or nuclear and cell surfaces of *in vitro* were wider than those in *in vivo* (Table 4). A comparison of nuclear and cell areas in *in vivo*, *in vitro* and callus tissues have been presented in Figures 3 and 4. Percentages of the nuclear and cell areas were observed to be increased from *in vivo* to *in vitro* samples when the surfaces were going up in the graph (Figure 3). Since mean nuclear and cell areas almost showed the same changes in the graphs from *in vitro* to *in vivo*, cell and nuclear sizes might have changed dependent on each other in different growth cultures (Figure 3). Although the NE callus showed a higher levels in low areas of nuclear and cell than E callus, mean nuclear and cell areas of E callus were more than NE tissue (Figure 4). Nevertheless, some mitotic abnormalities were also observed in mitotic division of *in vitro, in vivo* and callus. Along this line, such different mitotic irregularities were assessed as varied degrees of chromosome laggards and bridge, binucleate cells, asynchronous nuclei, cytomixis and micronucleuses (Table 5 and Figure 5). Non-embryogenic callus carried the most cytomixis and chromosomes laggards/bridge. Binucleate cells were observed in intact plant more than *in vitro* and in callus (Figure 5, i and l; Table 5). Some chromosomes showed sticky formations of chromosomes laggard and bridge which occurred in anaphase to telophase stages (Figure 5, f and j). Chromosomes laggard and non-oriented may produce micronuclei (Figure 5-g); if they fail to reach the poles in time to be included in the main telophase nucleus (Koduru and Rao, 1981; Utsunomiya *et al.,* 2002). Migration of chromatin material occurs through cytoplasmic connections originating from the pre-existing system of plasmodesmata formed within the tissues (Diaz Lifante *et al.,* 1992; Falistocco *et al.,* 1995). Chromatin/chromosome migration occurred in different directions from early prophase to telophase in the cytoplasmic studied cells. Chromosomes transfer from

Table 3. Mitotic behaviour of *Onobrychis viciifolia* in different growth conditions.[a]

Grow conditions	Interphase (%)	Prophase (%)	Metaphase (%)	Anaphase (%)	Telophase (%)	Mitotic Index (MI)
In vitro	73.16±0.21	21.02±0.06	3.00±0.11	1.34±0.01	1.46±0.01	26.84±0.58
In vivo	73.71±0.12	18.52±0.08	4.26±0.14	1.29±0.06	2.20±0.01	26.28±0.67
E[a]. callus	79.44±0.18	14.73±0.02	2.62±0.01	0.89±0.06	1.31±0.02	20.56±0.74
Non-E.callus	77.51±0.24	13.38±0.06	4.67±0.05	1.55±0.01	2.81±0.01	22.41±0.51

[a] Embryogenic, ±SD: Standard Division.

Table 4. The mean cells (C) and nuclei (N) area of *Onobrychis viciifolia* in different growth conditions.[a]

Grow condition	Cell (μm^2)	Nuclear (μm^2)	N/C
In vitro	$651.578^{b} \pm 6.21$	$141.777^{a} \pm 2.14$	0.217
In vivo	$493.174^{c} \pm 3.01$	$106.155^{c} \pm 1.24$	0.215
E.[b] callus	$745.327^{a} \pm 5.14$	$125.021^{b} \pm 2.41$	0.167
Non-E.callus	$619.802^{b} \pm 8.22$	$89.578^{d} \pm 1.15$	0.144

[a] The means of the populations with same small letters were not significantly different as per Duncan's multi-range test at $P< 0.05$. [b] Embryogenic.

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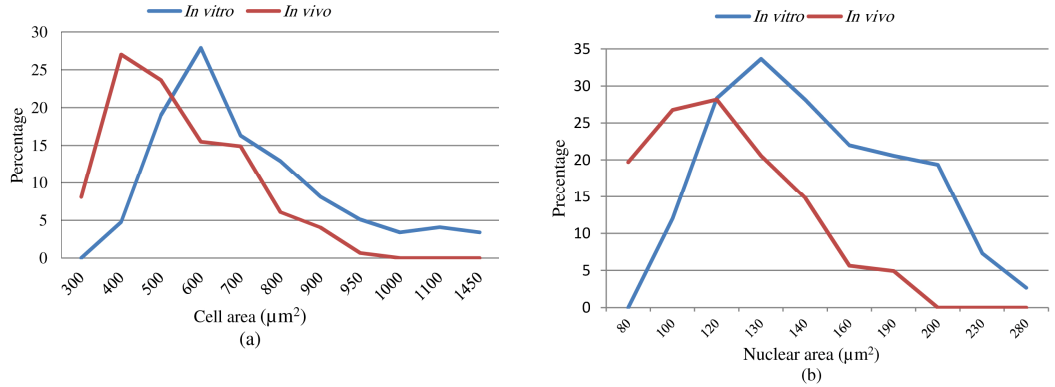

Figure 3. Distribution of cell and nuclear areas (µm^2) of *in vitro* and *in vivo* samples in prophase stage.

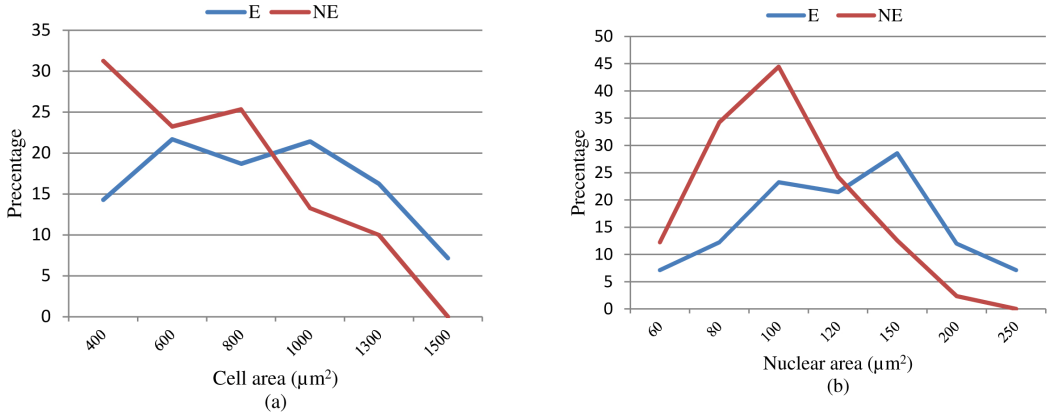

Figure 4. Distribution of cell and nuclear areas (µm^2) of embryogenic and non-embryogenic callus in prophase stage.

Table 5. Mitotic aberrations found of *Onobrychis viciifolia* in different growth conditions.[a]

Grow conditions	Cytomixis (%)	Bridge/Laggard (%)	Micronucleus (%)	Asynchronous nucleus (%)	Binucleate cells (%)
In vitro	-	0.53	0.42	0.49	0.97
In vivo	1.21	1.28	0.09	0.05	2.47
E. [a] callus	2.65	0.88	0.19	0.08	0.09
Non-E.callus	3.41	3.47	1.15	0.08	1.18

[a] Embryogenic, ±*SD*< 0.001.

cell to cell through cytoplasmic connections, an irregularity phenomenon known as cytomixis, has been just observed in *in vivo* growth culture and callus cells (Figure 5-k). Non-embryogenic callus showed non-dividing cells with small rounded nuclei, meristematic cells which were small with densely stained cytoplasm, prominent nucleus with condensed chromatin, and parenchyma cells which were large and elongated (Figure 5-e).

Karyotype parameters and Idiogram of the studied *Onobrychis* genus are illustrated in Table 6 and Figure 6 (a and b). Basic chromosome number and ploidy level were (x= 8) and (2n= 2x= 16), respectively. Mean value of the chromosome Long Arm (LA) varied from 0.64 to 1.67 µm. Average chromosome Short Arm (SA) differed from

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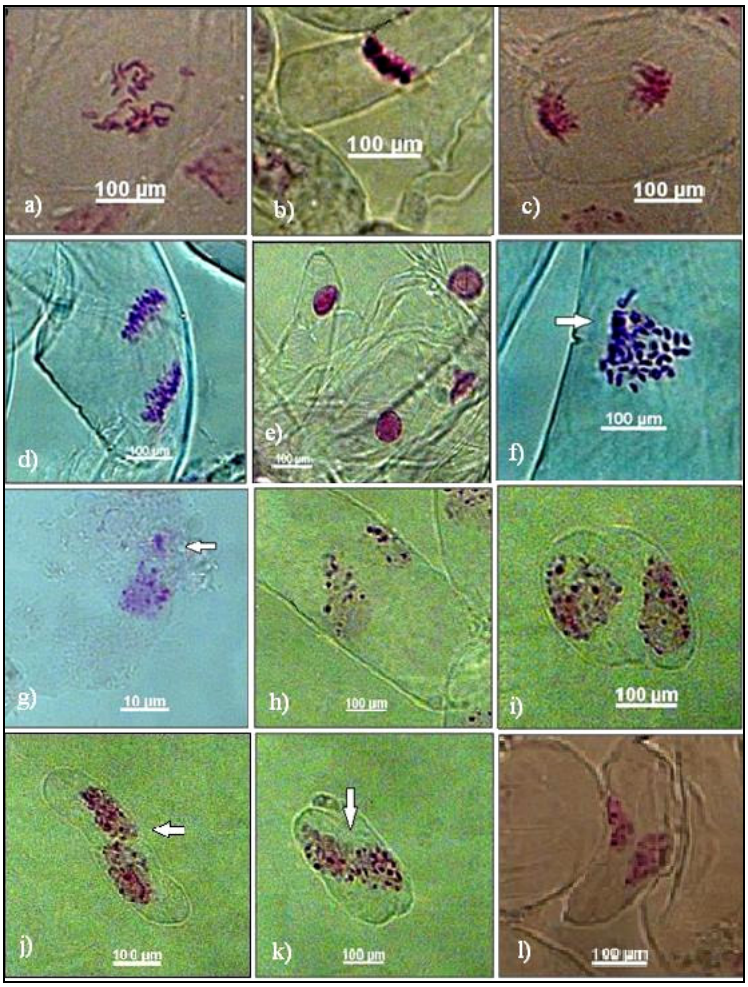

Figure 5. Representative normal and abnormal mitotic cells in *O. viciifolia* (2n=2x= 16). (a) Prophase; (b) Metaphase; (c) Anaphase; (d) Telophase; (e) Non-dividing cells; (f) Laggard chromosomes; (g) Micronucleus; (h) Asynchronous nuclei; (i) Binucleated cells; (j) Chromosomes bridge; (k) Cytomixis; (l) Binucleate cells, and Bars= 100 µm.

0.45 to 1.29 µm. Mean value of the Chromosome's Total Length (TCL) varied from 1.09 to 2.96 µm. Mean chromosome's Arm Ratio (AR) and Centromeric Index (CI) changed from 1.17 and 0.27 to 2.69 and 0.46, respectively (Table 6). The chromosomes were mostly median region (m) and sub-median region (sm) ones (Figure 6; Table 6). Karyotypic formula of *in vitro* and *in vivo* cells was 10 m+6 sm and one of a secondary constriction in the short arm of the second pair of chromosomes was observed. In terms of the Stebbins´ classification, the karyotypes fall into class *2B* with their *DRL* value amounting to 10.72 (Stebbins, 1971). The Total From percentage found to be 37.67% and the relative length of the shortest chromosome (S%) recorded 6.25% (Table 6). Scanning Electron Microscopic (SEM) study of root structure cells showed that *in vivo* sample had the enlarged cells with specific walls at the same arranged orientation, whilst *in vitro* cells looked different with non-aligned areas and unknown walls (Figure 7). Possibly, enough water, macro and micronutrients caused the non-distinctive cells in *in vitro* growth culture, while the in vivo cells should be adapted to the shortage of nutrient sources in the soil.

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Table 6. Karyological and chromosome analysis in *Onobrychis viciifolia*.[a]

Taxon	2n [a]	x [b]	DRL [c]	TF % [d]	VRC [e]	SC [f]	S% [g]	KF [h]
O. viciifolia	16	8	10.72	37.67	2.185	2B	6.25	10 m [i] +6 sm [j]
Chromosome no.	SA (µm) [k]	LA (µm) [l]	TCL (µm) [m]	AR [n]	CI [o]		RL% [p]	Sat [q]
1	1.29	1.67	2.96	1.29	0.44		16.97	-
2	0.93	1.84	2.77	1.98	034		15.88	+
3	1.11	1.44	2.54	1.30	0.44		14.62	-
4	0.91	1.51	2.42	1.66	0.38		13.87	-
5	0.64	1.72	2.36	2.69	0.27		13.53	-
6	0.77	0.90	1.67	1.17	0.46		9.57	-
7	0.47	1.15	1.62	2.45	0.29		9.28	-
8	0.45	0.64	1.09	1.42	0.41		6.25	-
As K% [r]	A [s]	A_1 [t]	A_2 [t]	SI % [u]	CG % [v]	DI % [w]	Syi % [x]	Rec % [x]
62.32	0.245	0.376	0.277	36.82	32.42	8.98	60.44	73.64

[a] Somatic chromosome number, [b] Basic chromosome number; [c] Difference of Relative Length; [d] Total Form percentage; [e]Value of Relative Chromatin; [f] Stebbins' symmetry Classes, [g] Relative length of shortest chromosome. [h] Karyotype Formula; [i] Median region; [j] Sub-Median region; [k] SL: Short Arm; [l] Long Arm; [m] Total Chromosome Length; [n]Arm Ratio; [o] Centromic Index; [p] Relative Length percentage, [q] Presence (+) or absence (-) of satellite; [r] Arano method; [s] Classification of karyotype symmetry; [t] Intrachromosome asymmetry index; [u] Symmetry Index percentage, [v] Centromeric Gradient; [w] Dispersion Index percentage; [x] Indices of karyotype symmetry.

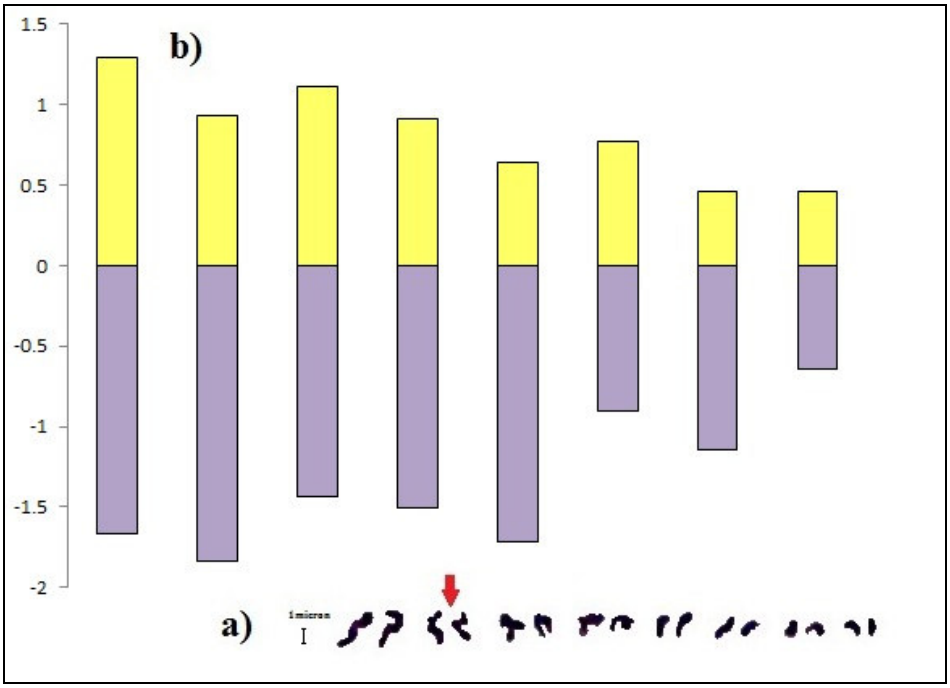

Figure 6. a) Karyogram and b) Ideogram of *Onobrychis viciifolia*. Arrow indicates the chromosome pair with secondary constriction. Bar= 1µ.

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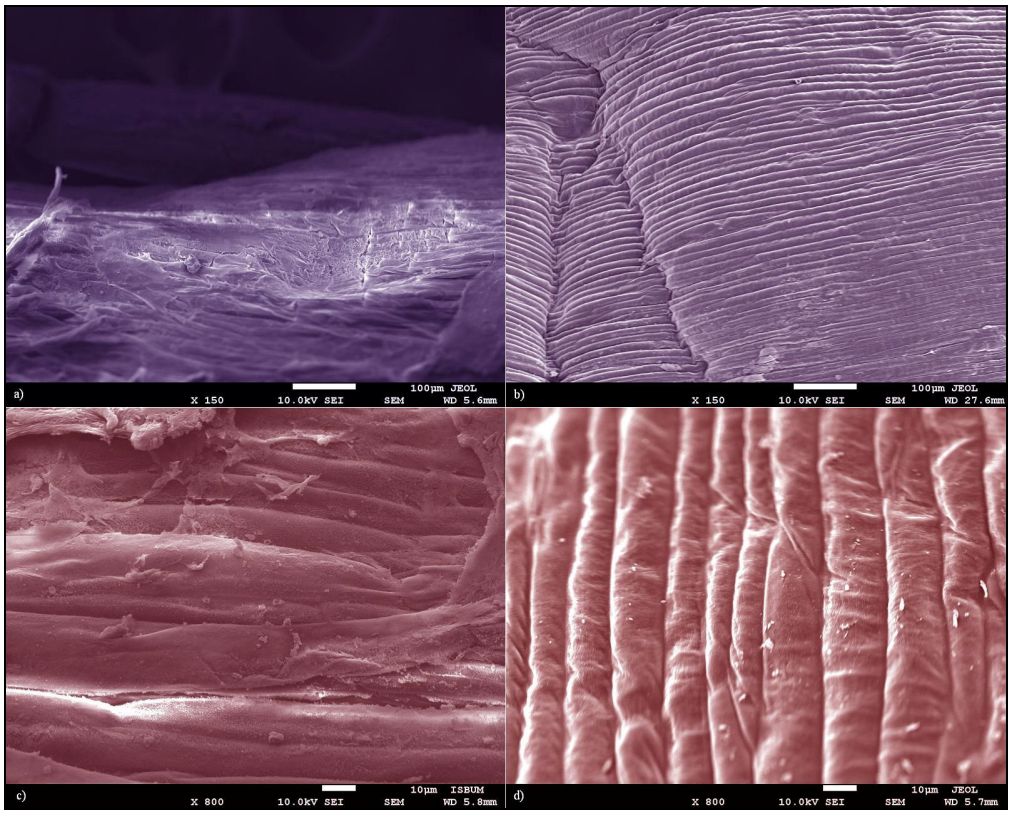

Figure 7. Scanning electron micrographs showing root surface cells in *in vitro* (a) and (c) and *in vivo* (b) and (d)

DISCUSSION

Unfortunately, there is little information on genomic and genetic aspects of *Onobrychis viciifolia*. The present investigation was a first attempt to record cytological study as well as mitotic abnormality of *in vitro*, *in vivo*, embryogenic and non-embryogenic callus.

Capability of performing wide ranges is an advantage of mitotic investigations for chromosomal variation analysis including polyploidy (Otto and Whitton, 2000). Consequently, cytogenetic maps and evolutionary relationships among species can be constructed based on cytological information (De Wet, 1979; Stevens and Bougourd, 1991; Fukui and Nakayama, 1996; Seijo and Fernandez, 2001). Achievements of the current study were not in conformity with the results obtained by Thomas and Davidson (1983). They stated that the relationship between nuclear and cell sizes can change abruptly without the effect of the mitotic activity, while direct changes in mitotic division, nuclear and cell areas were observed from *in vitro* to *in vivo* growth culture in this study. Consistent with assessment results of the present study, Moghaddam and Taha (2008) reported the increment of chromosome numbers and reduction of *MI* value from *in vitro* and *in vivo* to callus tissue, which might be referred to cellular unsteadiness of cells in callus tissues. Taha and Wafa (2012) hypothesized that a combination of auxin and cytokinin hormones would affect the regulation mechanisms of nuclear and cell areas in callus, which was observed in this study as well (Figures 3 and 4).

C-value standard samples, which have been previously provided by Dolezel *et al.* (2007), were employed to define and

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compare the DNA content of *O. viciifolia*. Two basic chromosome numbers (x= 7 and x= 8) and three ploidy levels (2n= 2x= 14, 2n= 4x= 28, 2n= 8x= 56 and 2n= 2x= 16, 2n= 4x= 32) have been presented in the genus *Onobrychis* (Abou-El-Enain, 2002). One ploidy level (2n= 4x = 28) has previously been reported by Takhtajan (1990), and Hesamzadeh and Ziaie (2010) in *O. viciifolia*, while three diploid *O. viciifolia* accessions with 14 chromosomes were recently distinguished (2n= 2x= 14) by Hayot Carbonero *et al.* (2013). Ghanavati *et al.* (2012) stated that *x*= 8 was observed only in the tetraploid genus of *Onobrychis*.

Unlike the previous scientific researches, image analysis showed a diploid variety of *O. viciifolia* (2n= 2x= 16) rather than the reported tetraploid verities (2n= 4x= 28) by (Yan-Jun *et al.,* 2006; Hesamzadeh and Ziaie, 2010; Ranjbar *et al.,* 2010). Goldblatt (1981) hypothesized chromosome *x*= 7 was indeed derived from *x*= 8 which mutated hereditarily in the genus. He also suggested that *x*= 8 is ancestral in the genus and those species of *x*= 7 are derived through aneuploid loss. Therefore, the studied variety has probably originated from a wild *Onobrychis* genus which has mutated and finally adapted to the environmental tensions. This variety is already harvested for forage purposes due to high seed quality and substantial dry matter yield. The genesis provenance of *Onobrychis* has been stated to refer to the Mediterranean countries by Ashurmetov and Normatov (1998), although some other researchers believe this debatable species is originated from south eastern Asia (Yildiz et al., 1999; Ranjbar et al., 2009). Since a distribution of the most diploid species is in south western Asia due to the temperature influence (Ranjbar *et al.,* 2010), variation of *viciifolia* is attributed to this region particularly to Iran.

Cytological researches on the *Onobrychis* genus have been focusing mainly on the chromosome count (Baltisberger, 1991; Karshibaev, 1992; Slavivk *et al,* 1993), whilst a few studies have been concerned with karyological criteria being carried out for taxonomic objectives (Khartoum *et al.,* 1991; Mesicek and Sojak, 1992, Ranjbar *et al.,* 2010). In reality, sainfoin is highly influenced by ecological factors, since it is an open pollinated plant and changes with variations in the growing aspects in subgenuses of *Onobrychis* (Hesamzadeh and Ziaie, 2010).

Karyotypic asymmetry is explained by either shifting of the centromeric position from median to subterminal or through differences in relative size between individual chromosomes. Types of chromosomes in all the previous populations of *O. viciifolia* were located in Srebbines Classes (SC) *1A* and *2A* (Hesamzadeh and Ziaei, 2010), whilst the chromosome set of the studied variety was *2B*. The Dispersion Index (DI), which was introduced by Lavania and Srivastava (1992), to assess the phylogenetic differentiation, is the proportionate measure of the Centromeric Gradient (CG). Higher values of *DI* are considered to indicate higher levels of karyotype specialization. In the studied species of *O. viciifolia*, it helped to evaluate the chromosome set with 8.98%. The values of the A_1 intrachromosomal index parameter of the studied population was similar to some reported accessions by Hesamzadeh and Ziaei (2010).

Cell division disturbance is normally caused by the initial effect on the mitotic process (Hess 1999). The most common types of aberrations, in the present study, were the chromosomes irregular in the equatorial plate such as cytomixis, Chromosome Bridge and binucleate cells which occurred in different phases of the mitotic. These abnormalities might be attributed to failure in functionality of the DNA spindle rather than prohibition of spindle fibers (Grant, 1978; Mansour, 1984). Imperfect separation of homologous chromosomes is occurring even in the presence of sprinkling fibers and hence semi-connectivity of Chromosome bridge results from suck stickiness phenomenon (Aksoy *et al.,* 2008).

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Ultimately, it is concluded that nucleus volume increment and endoreduplication, can occur as gene expression, are changed by cultural ambient (Gaspar *et al*. 2000). Apart from changes in biochemical behaviour, main impacts of the attended changes presumably can be titled as misprinted cell plates, partial cell cycle division and insufficient cell wall formation, cell junction loosening, generation of a non-proliferative cell with enormous nucleus, which cause loss of capability of collaborating with other cells (Verma, 2001).

Abbreviations

Embryogenic (E), Non-Embryogenic (NE), Scanning Electron Microscopy (SEM), DNA Constancy value (C-value); Integrated Optical Density (IOD), Long Arm (LA), Short Arm (SA), Total Length (TL), Arm Ratio (AR).

ACKNOWLEDGEMENTS

The authors would like to thank the University of Malaya, Malaysia for the facilities and financial support provided (IPPP Grant PV025/2011B and UMRG grant RP025-2012A).

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بررسی روی سیتولوژی و کاریوتایپ یک واریته جدید اسپرس در کشت این ویوو و این ویترو کالوس

ص. مهاجر و ر. م. طه

چکیده

اسپرس با ۱۳۰ گونه یک ساله و چند ساله یکی از مهمترین گیاهان علوفه ای می باشد. در این تحقیق، رفتار سلولی در کالوس با القای جنین و بدون القای جنین و همچنین بررسی تغییرات در مریستم ریشه

[DOR: 20.1001.1.16807073.2014.16.7.6.2]

حاصل از محیط کشت این ویوو و این ویترو مورد بررسی قرار گرفت. برخلاف مطالعات گذشته، یک واریته دیپلوییید اسپرس (*Onobrychis viciifolia* Scop.) با ۱۶ کروموزوم برای اولین بار مشاهده شد. با اینکه بطور کلی تقسیم میتوز در شرایط مختلف کشت منظم بود، اما یکسری تقسیم های نامنظم بویژه در کالوس بدون القای جنین مشاهده شد. نسبت سطح هسته به سلول در کالوس کمتر از این ویوو و این ویترو بود و سطوح مختلف پلی پلوئیدی فقط در سلول های کالوس مشاهده شد. اکثر سلول های این ویوو و این ویترو در مرحله (G_1) اینترفاز در مقایسه با مراحل سنتز و (G_2) مشاهده شدند. کاریوتایپ به صورت ۱۰ کروموزوم متاسنتریک و ۶ کروموزوم ساب متاسنتریک ترسیم و همچنین یک فرورفتگی ثانویه در بازوی کوچک جفت کروموزوم دوم تشخیص داده شد. در نهایت، میکروسکوپ الکترونی برای بررسی تفاوت شمای سلول های این ویوو و این ویترو مورد استفاده قرار گرفت.

[DOR: 20.1001.1.16807073.2014.16.7.6.2]