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Table of Contents

Journal of Cell and Molecular Research (2011-2012) 3 (2), 43-50

Ferula assafoetida latex can cause damage to bacterial DNA

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Abstract

Asafoetida is the dried latex exuded from the living underground rhizome or tap root of *Ferula assafoetida*. Antibacterial characteristic of asafoetida was shown using the circular zone diameter of bacterial growth inhibition by disk-diffusion method on two gram positive and three gram negative bacteria. Then, the bacterial genomic DNA damage, induced by *F. assafoetida* latex, was demonstrated using the comparison of random amplification of polymorphic DNA profiles generated by polymerase chain reaction of control and treated bacterial genomes. The results showed that the number of primers that produced bands in each bacterium were higher in control samples compared to those treated with asafoetida. This and the absence or presence of bands between controls and treatments confirm rearrangements and DNA damage in the priming binding sites of bacterial genome.

***Keywords*:** Antibacteria, Asafoetida, DNA Polymorphism, DNA damage

Introduction

Asafoetida (Angedan in Persian), is the dried latex (gum oleoresin) exuded from the living underground rhizome or tap root of *Ferula assafoetida* (Apiaceae family), which is a perennial herb (1 to 1.5 m high). The species is native to Persia (Iran) and India. Typical asafoetida contains about 40-64% resin, 25% gum, 10-17% volatile oil, and 1.5-10% ash. The resin portion is known to contain asaresinotannol, ferulic acid, umbelliferone and four unidentified compounds (Singhal et al., 1997). Asafoetida has a broad range of uses in traditional medicine as an antimicrobial, with well documented uses for treating chronic bronchitis and whooping cough, as well as reducing flatulence (Srinivasan, 2005; Rahman et al., 2008).

The disk-diffusion method is more suitable for routine testing in a laboratory where a large number of isolates are tested for susceptibility to numerous antibiotics. Growth of the organism and diffusion of the antibiotic commence simultaneously resulting in a circular zone of inhibition in which the amount of antibiotic exceeds inhibitory concentrations (Barker and Kehoe, 1995; Ottaviani et al., 2001).

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Random amplification of polymorphic DNA (RAPD) is used extensively for species classification and phylogenetic analysis (Franklin et al., 1999; Yamamoto et al., 2001; Sudupak et al., 2002; Ronimus et al., 2003; Krizova et al., 2008). A novel application of RAPD method is as biomarker assay to detect DNA damage and mutational events for example rearrangements point mutation, small insert or deletions of DNA and ploidy changes in cells of bacterium, yeast, plant, and animal (Jones and Kortenkamp, 2000; Atienzar and Jha, 2002; De Wolf et al., 2003; Atienzar et al., 2004; Castano and Becerril, 2004; El-Sherbeny et al., 2005; Shahrtash et al., 2010).

The aim of this study was to detect bacterial DNA (gram positive and gram negative) damage induced by *Ferula assafoetida* latex, using the RAPD technique. Detection of genotoxic effect involves the comparison of RAPD profiles of DNA generated by control and treated bacteria.

Materials and Methods

Microbiological culture and plant material

Five bacteria samples were taken from Persian Type Culture Collection (PTCC) of Iranian Research Organization for Science and Technology. They included three gram negative bacteria *Pseudomonas putida* (PTCC 1694), *Pseudomonas aeruginosa* (PTCC 1181) and

Xanthomonas campestris (PTCC 1473) and two gram positive bacteria *Staphylococcus aureus* (PTCC 1431) and *Bacillus subtilis* (PTCC 1156). The bacteria (100 µL of each bacterial suspension with 0.5 McFarland concentrations) were grown in petri dishes which contained a layer of agar-based Müller-Hinton growth medium. Once the growth medium in the petri dish was inoculated with the desired bacteria, the plates were incubated at the temperature of 37°C for 24 h (Atlas, 2004).

Asafoetida was obtained by abrasion of *F. assafoetida* rhizome. The plants are grown naturally in the Fars province. The asafoetida was used with 100 mg/ml concentration.

Antibacterial disk-diffusion method

The antibiotic disks (6.4 mm of diameter) that contained 10 µL of *F. assafoetida* latex (100 mg/ml concentration) or positive control antibiotics (Gentamicin against gram-negative and Penicillin against gram-positive bacteria) were placed on the agar plates streaked with bacterial suspension and inside a laminar airflow system. The antibiotic activity was measured using the circular zone diameter (mm) of bacterial growth inhibition by disk-diffusion method.

DNA extraction and RAPD experiment

Total genomic DNAs of the bacteria were extracted using GenElute Bacterial Genomic DNA kit (Sigma). The PCR amplification was carried out using twenty three 10-base pair random primers (Eurofins MWG Operon-company) with control and asafoetida treated bacterial genomic DNA as the template. PCRs were performed in a reaction mixture of 20 µl containing approximately 80 ng of the genomic DNA dissolving in sterile distilled water, 10X PCR buffer (2 µL), 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 2 µl of 10 µM primer and 1 U Taq DNA polymerase. The RAPD protocol consisted of an initial denaturing step of 5 min at 94°C, followed by 35 cycles at 94°C for 1 min (denaturation), 37°C for 1 min (annealing), and 72°C for 2 min (extension) with an additional extension period of 10 min at 72°C. The PCR amplification products were separated on 1% agarose gel using Tris-Borate-EDTA (TBE) buffer and GeneRuler 100bp DNA ladder (Fermentas, Germany). All the PCR examinations were carried out by Bioer XP thermal cycler.

Results

Table 1 shows the circular zone diameter (mm) of bacterial growth inhibition by disk-diffusion method for *F. assea-fotida* latex, Gentamicin and Penicillin. The circular zone diameters of positive and negative controls were slightly higher than *F. asseafotida* latex.

Table 2 shows the nucleotide sequences of the eighty 10-mer primers which produced bands from twenty three examined primers by RAPD analysis. Table 3 is the summary of RAPD products obtained from five examined bacteria under control and asafoetida treated conditions. Total number of bands amplified from primers in each bacterium under normal and stress condition were 68 to 123. In addition, average numbers of polymorphic bands per each primer were 0.7 to 4.7 percentages. Figures 1 to 5 are RAPD profiles of genomic DNA from five examined bacteria under control and *F. assafoetida* latex treatment.

Table 1. Circular zone diameter (mm) of bacterial* growth inhibition by disk-diffusion method.

Sample	1	2	3	4	5
F. assea-fotida latex	19	15	21	18.2	17
Gentamicin#	20.3	20.1	22.1	-	-
PenicillinΦ	-	-	-	22	21

*1- *P. Putida* 2- *P. Aeruginosa* 3- *X. compestris* 4- *S. aureus* 5- *B. subtilis*
#Gentamicin as positive control against gram-negative bacteria
ΦPenicillin as positive control against gram-positive bacteria

Table 2. Nucleotide sequences of the eighty 10-mer primers* which produced bands by RAPD analysis.

Primer	Nucleotide sequence (5´-3´)
1. OPA-02	TGCCGAGCTG
2. OPA-07	GAAACCGGTG
3. OPA-08	GTGACGTAGG
4. OPA-09	GGGTAACGCC
5. OPB-07	GGTGACGCAG
6. OPB-08	GTCCACACGG
7. OPD-02	GGACCCAACC
8. OPD-03	GTCGCCGTCA
9. OPD-04	TCTGGTGAGG
10. OPD-05	TGAGCGGACA
11. OPN-01	CTCACGTTGG
12. OPN-02	ACCAGGGGCA
13. OPN-03	GGTACTCCCC
14. OPN-04	GACCGACCCA
15. OPN-06	GAGACGCACA
16. OPN-08	CCTCCAGTGT
17. OPC-14	TGCGTGCTTG
18. OPF-14	GGTGCGCACT

*All primers were provided by Eurofins MWG Operon-company (Ebersberg, Germany).

Table 3. Summary of RAPD products obtained from five examined bacteria* under control and stress.

	1		2		3		4		5	
Parameter	C	S	C	S	C	S	C	S	C	S
Total number of primers examined	23	23	23	23	23	23	23	23	23	23
Number of primers that produced bands	18	16	18	15	18	16	18	14	18	17
Total number of bands amplified from primers	86	89	123	109	120	68	117	81	106	71
Average number of bands per each primer	4.7	5.6	6.8	7.2	6.6	4.3	6.5	5.8	5.8	4.2
Total number of polymorphic bands	34.5	36	58.2	33	67.5	11	66	24	84.5	39
Average number of polymorphic bands per each primer	1.9	2.3	3.2	2.2	3.8	0.7	3.6	1.7	4.7	2.3
Polymorphic bands percentage from total bands	40.1	40.4	47.3	30.3	56.3	16.2	56.4	29.6	79.7	54.9

*1- *P. Putida* 2- *P. Aeruginosa* 3- *X. compestris* 4- *S. aureus* 5- *B. subtilis*

Discussion

In this study, the gram negative bacteria *P. putida*, *P. aeruginosa* and *X. campestris* and the gram positive bacteria *S. aureus* and *B. subtilis* were used for investigating the effect of *F. assafoetida* latex on bacterial DNA. Asafoetida has a broad range of uses in traditional antimicrobial therapy (Srinivasan, 2005) but mechanism of its function on bacteria is not clear. In this experiment, antibacterial characteristic of asafoetida was studied using the circular zone diameter (mm) of bacterial growth inhibition by disk-diffusion method and by RAPD profiles of their genomic DNA. The diameter of the inhibition zone is a function of the

amount of drug in the disk and susceptibility of the microorganism. The circular zone diameters of bacterial growth inhibition in five examined bacteria were from 15 to 21 mm which confirm high inhibition of asafoetida on bacterial growth (table 1).

Out of the 23 oligonucleotide primers tested, only 18 primers gave specific and stable results and 14 to 17 primers indicated changes in the RAPD profiles following asafoetida treatment. As table 3 shows the number of primers that produced bands in each bacterium were higher in control samples compared to the asafoetida treatment. This confirms that *Ferula assafoetida* latex may cause changes in the bacterial DNA. In addition, the comparison of RAPD profiles of genomic DNA from five bacteria between control and *F. assafoetida* latex treatment demonstrate obvious variations. Meanwhile, the primers gave a total of 970 bands ranging from 180-3000 base pairs on gel agarose electrophoresis (figure 1 to 5). Bacterial DNA damage was shown by RAPD profiles via absence or presence of bands. The total number of RAPD bands on profiles of asafoetida treatments compared to the total control bands was 276 to 143, respectively. Disappearing bands are likely to be due to changes in oligonucleotide priming sites, originated from rearrangements and DNA damage in the priming binding sites (Nelson et al., 1996; Enan, 2006; Liu et al., 2009; Shahrtash et al., 2010). Structural changes or some changes in DNA sequence due to mutation and/or large deletions (bringing two pre-existing annealing sites closer) were created in new priming sites. Our findings and some other reports support the idea that bacterial DNA polymorphisms detected by RAPD can be considered as a biomarker assay for detection of the genotoxic and DNA damage effects of natural or synthetic material with antibacterial property (Kumar et al., 2004). Previous studies had shown that changes in DNA fingerprint offered a useful biomarker assay in toxicology (Savva, 1996 and 1998).

There is no obvious relationship between the circular zone diameters of bacterial growth inhibition (table 1) and the DNA damage parameters (table 3), indicating the possible involvement of other mechanisms in the inhibition process.

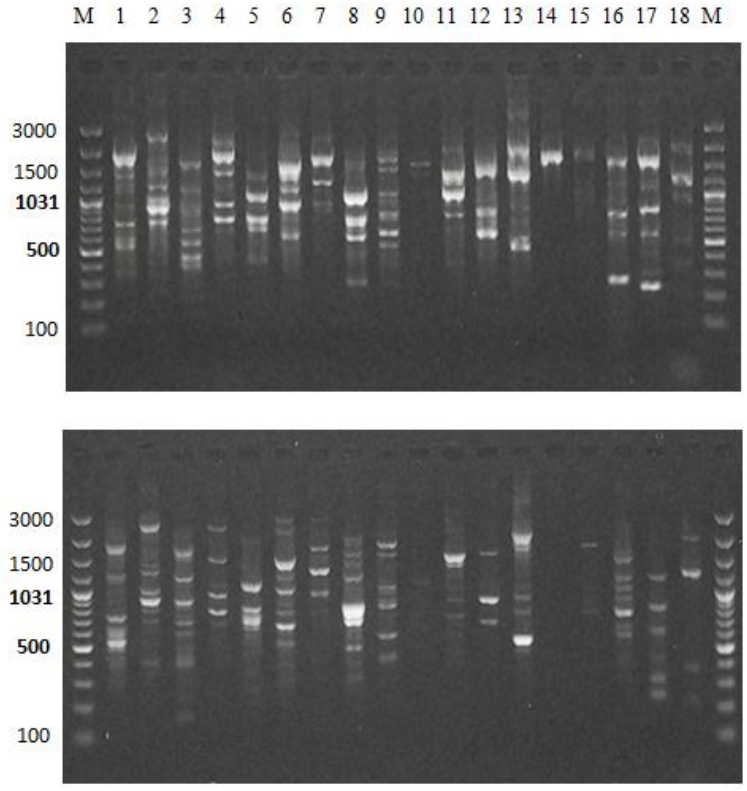

Figure 1. RAPD profiles of genomic DNA from *P. Putida*. Control (top) and *F. assafoetida* latex treatment (down). M: 100 bp DNA ladder (100-3000) and 1 to 18 are primers as showed in table 1.

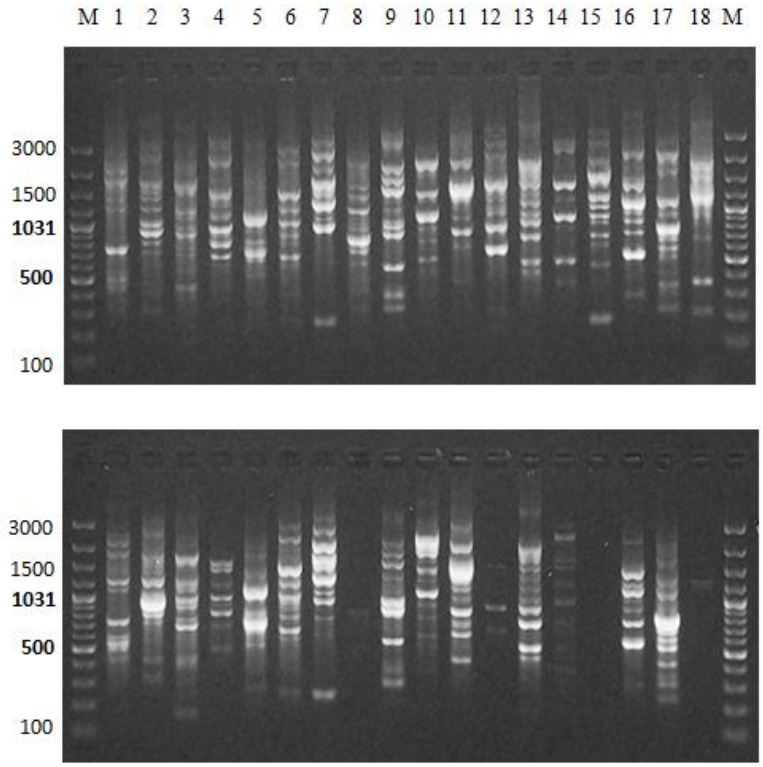

Figure 2. RAPD profiles of genomic DNA from *P. Aeruginosa*. Control (top) and *F. assafoetida* latex treatment (down). M: 100 bp DNA ladder (100-3000) and 1 to 18 are primers as showed in table 1.

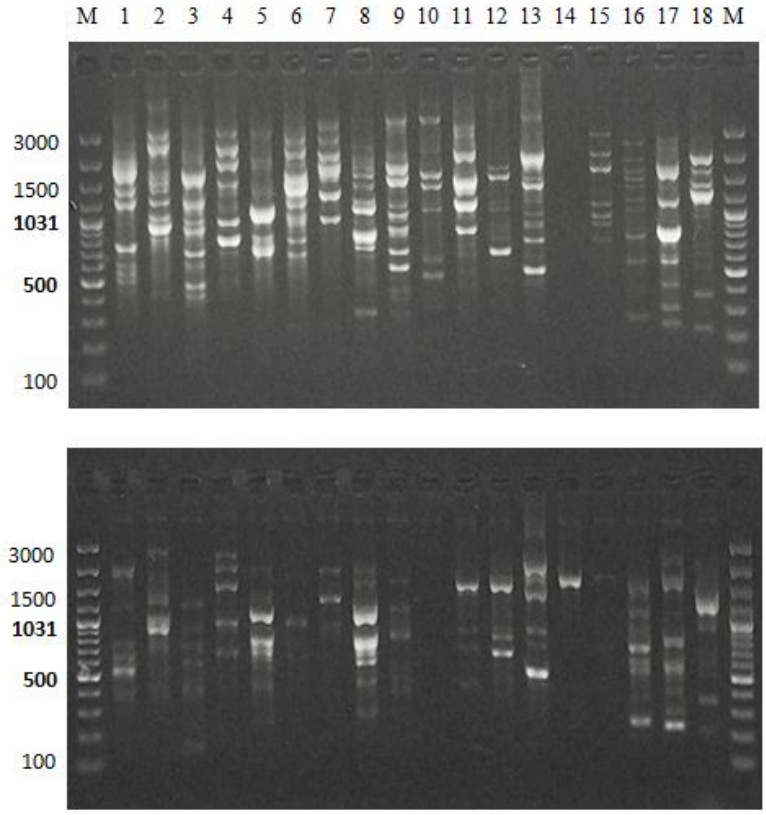

Figure 3. RAPD profiles of genomic DNA from *X. compestris*. Control (top) and *F. assafoetida* latex treatment (down). M: 100 bp DNA ladder (100-3000) and 1 to 18 are primers as showed in table 1.

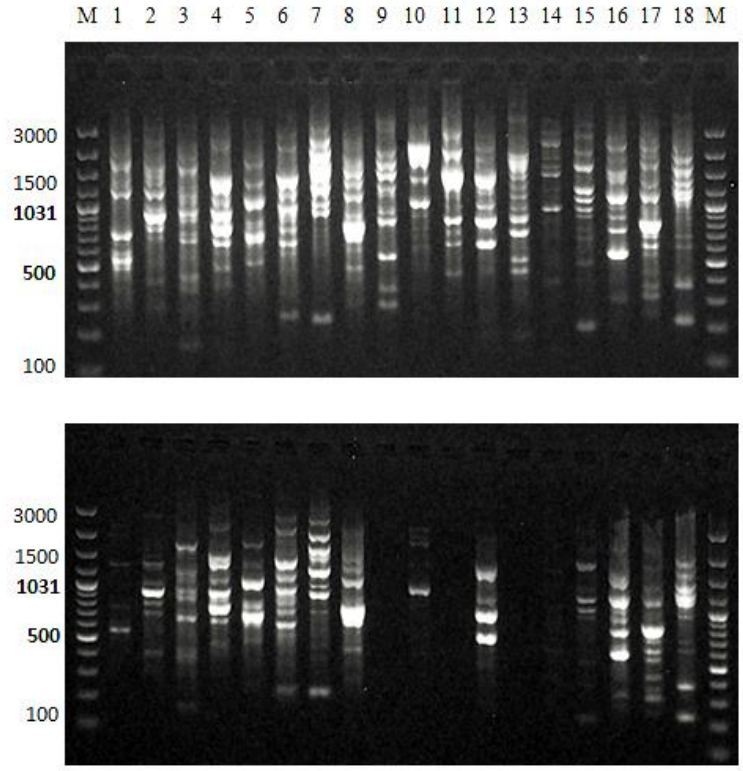

Figure 4. RAPD profiles of genomic DNA from *S. aureus*. Control (top) and *F. assafoetida* latex treatment (down). M: 100 bp DNA ladder (100-3000) and 1 to 18 are primers as showed in table 1.

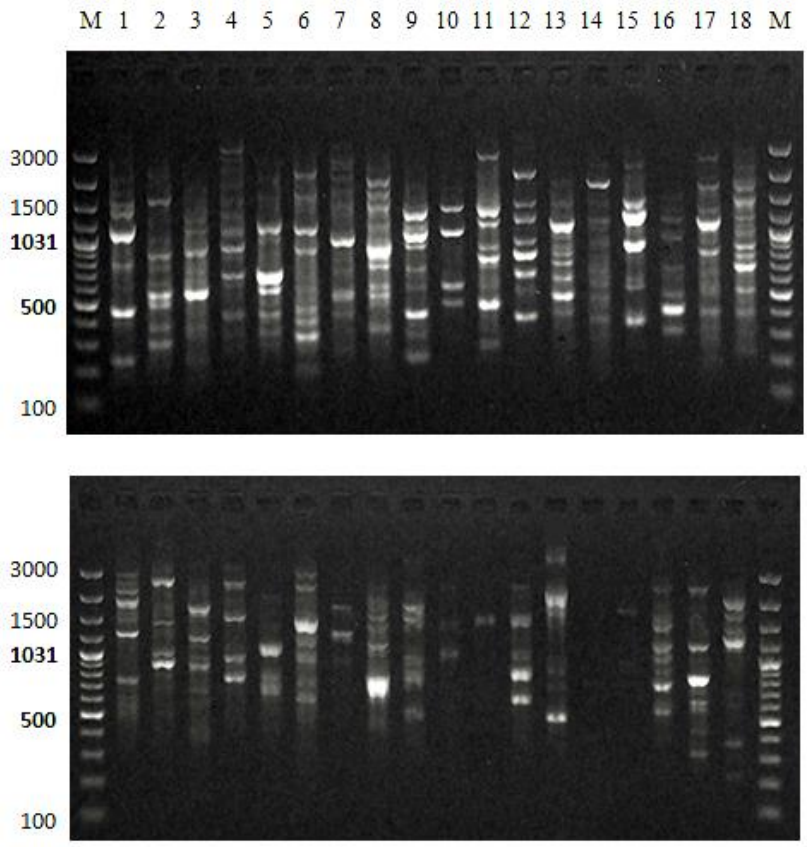

Figure 5. RAPD profiles of genomic DNA from *B. subtilis*. Control (top) and *F. assafoetida* latex treatment (down). M: 100 bp DNA ladder (100-3000) and 1 to 18 are primers as showed in table 1.

Acknowledgment

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Cytotoxic effect of essential oils from *Salvia leriifolia* Benth. on human Transitional Cell Carcinoma (TCC) and mouse fibroblast

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Abstract

Essential oils, with plant origin, have been of special attention in cancer research during recent years. Despite many reports on cytotoxic effects of plants from genus salvia, the potential application of their extracts in cancer therapy remains to be assessed in more precise and detailed examinations on the main cause of such effects. In this research, the cytotoxic effect and anticancer activity of essential oils from *S. leriifolia* on human Transitional Cell Carcinomaa (TCC) were studied *in vitro*. The antiproliferative activity of essential oils on TCC and L929 (control) cells was determined by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay, by which the mitochondrial dehydrogenase enzyme activity is assessed based on reduction of the MTT to purple. The amount of essential oils to induce 50% of cells to die, designated as IC_{50}, was determined by repeated experiments and application of different doses of the essence. The established IC_{50} on TCC cells for the essences extracted in two different years of 2006 and 2008 and from two locations of Bajestan and Neyshabour was respectively as: 466 and 250 µg/ml, and 233 and 212 µg/ml. *S. leriifolia* essential oil did not show any detectable effect on L929 cells in this range of concentration. *S. leriifolia* essential oil has inhibitory effects on the growth of both TCC and normal L929 cell lines, although the effective concentrations were significantly different in these cell lines. This effect was dose dependent.

Keywords**:** *S. leriifolia,* essential oil, cytotoxic, MTT

Introduction

Essential oils are traditionally known for their antiviral, antibacterial, and antifungal properties. In recent years, however, a great deal of attention has been directed to their capacity in the field of cancer research (Jalili et al., 1999; Young, 2005).

In search for better sources of essential oils, *S. leriifolia,* growing mainly in south and hot regions of Khorassan and Semnan provinces, I. R. Iran (Jones, 2009) seems to be a good candidate, due to its valuable pharmacological significance as anti hyperglycemia, anti-inflammatory, analgesic, muscle relaxation, anti-oxidant and sedative effects (Feizzadeh, 2008; Hosseinzadeh and Arabsanavi, 2001; Hosseinzadeh et al., 2009; Jackson, 2009).

S. leriifolia leaves are rich for a special chalcon, butein, with known inhibitory effects on protein kinases. Butein is a very important component in controlling of cell division and differentiation process, and induces apoptosis in human leukemic HL-60 cells. The caspase-3 activity was increased significantly following butein-induced apoptosis (Russin et al., 1989). Despite convincing evidences of considerable content of butein in *S. leriifolia*, there is not enough investigation on antitumor, anticancer, cytotoxic and antiproliferative properties of this plant. In this project the cytotoxic activities of essential oils from *S. leriifolia*, collected from different locations (Bajestan and Neyshabour) and time periods (2006, 2008) were examined on TCC cells in comparison with L929 as control cells.

Materials and Methods

Essential oil extraction and preparation

Essential oils were extracted from *S. leriifolia* leaves by hydrodistilation as previously described and preserved in freezing temperature of about -20ºC (Rustaiyan et al., 2007)

Cell culture

The extracts of essential oils were dissolved in dimethyl sulfoxide (DMSO), (Merk, Germany) and diluted in Dulbecco's modified eagles medium (DMEM) supplemented with 10% fetal calf serum

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(FCS) to reach different working concentrations. Two cell lines, TCC and L929, were cultured in DMEM, supplemented with 10% fetal calf serum (FCS), (Gibco, Scottland) at 37ºC in a humidified atmosphere containing 90% air and 10% CO_2.

Preparation of solutions with different concentrations of essential oils

In order to prepare solutions with different concentrations of essential oils, 50 µl (based on the calculated density of the solution, 1 µl equals to 1 µg) of the essences were dissolved in 950 µl DMSO and culture medium, and used as stock solution.

Different concentrations (80, 160, 320, 640 and 1300 µg/ml) were prepared by diluting the stock solution in proper volumes of the culture medium.

Cytotoxicity assay

Assessment of the cell viability was carried out by the MTT assay (Mossman, 1983) using 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (sigma, Deisenhofen, Germany). This assay is based on the metabolic reduction of soluble colored formazan product, which can be determined spectrophotometrically after dissolving in DMSO. Briefly, the cells were cultured in T_{25} and T_{75} flasks, and after 90% confluency, they were removed from the culture dishes by trypsinization and suspended in 10 ml culture medium, and seeded with a cell counts of 8×10^3 for TCC and 5×10^3 for L929 cells per well in 96-well plates. The final volume of each well reached to 200 µl by adding the culture medium. The cells were left to grow for 24 to 48 h, and they were then exposed to different concentrations of essential oils (0, 80, 160, 320, 640 and 1300 µg/ml). The MTT assay was performed 24, 48 and 72 h after the treatment. For this, 5 mg/ml of fresh and sterilized MTT dye, in Phosphate-buffered saline (PBS), was prepared. 20 µl of the MTT solution was added to each well and the plates were incubated at 37ºC for 4 h. During this period, the living cells produced a blue, insoluble formazan from the yellow, soluble MTT. The remaining MTT solution was removed and 200 µl of DMSO was added to each well to dissolve the formazan crystals. Absorbance for each well was measured at 570 nm (single wavelength) using an ELIZA plate reader. All experiments were performed in triplicates. Cells were treated with various concentrations of essential oils observed under a light-inverted microscope for morphological alterations after 48 h.

Statistical analysis

The data were analyzed statistically using PRISM, INSTAT and SPSS software's. The significant level was ascertained by one way analysis of variance (ANOVA), followed by Tukey multiple comparison tests. Results were expressed as the mean ± SD. P values of <0.05, <0.01, <0.001 in the Tukey test were considered as significant.

Results

The microscopic observations indicated that the TCC cells altered their morphology 48 h after treatment with the essential oils (figure 1).They turned to round shape and nuclear pigmentations happened. These changes were intensified by the time and increasing the concentrations of the extracts. The percentage of living cells in the treated cultures against the control ones was calculated using the following formula:

Living cells (%): (absorbance of the treated cells in each well / mean absorbance of the control cells) × 100.

The dose-responsive curves were calculated at different concentrations of the essential oils and expressed as the mean percentage fraction of control ± standard division (SD). The amount of essential oils to induce 50% of the cells to die, called IC_{50}, was determined by repeated experiments and the application of different doses of the essence.

The most significant effect of apoptotic induction occurred at 48 h after the essential oils administration. The established IC_{50} values, after this period of time, in TCC cells treated with extracts from plants collected in two different years of 2006 and 2008 and from two locations of Bajestan and Neyshabour were respectively as: 466 and 233 µg/ml; and 250 and 212 µg/ml. The essential oil from plants of Neyshabour (2008) showed the highest antiproliferative activity. There was a significant difference between cell viability of TCC and L929 in all examined concentrations (figure 2):

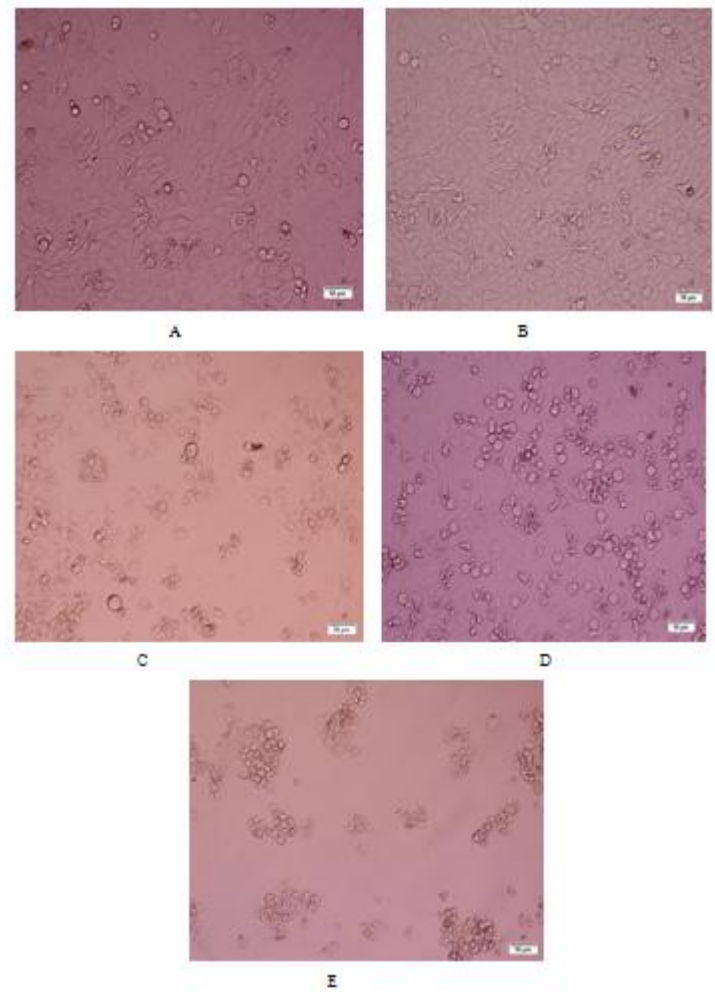
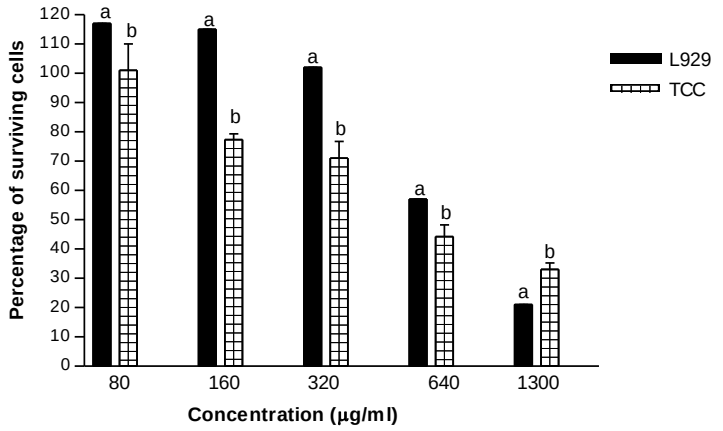


Figure 1. Micrographs of the TCC cells cultured for 48 h and under conditions of (A) without any treatment, and treated with (B) 160 µg/ml, (C) 320 µg/ml, (D) 640 µg/ml, and (E) 1300 µg/ml of essential oil which extracted from plants of Neyshabour (2008) collections.



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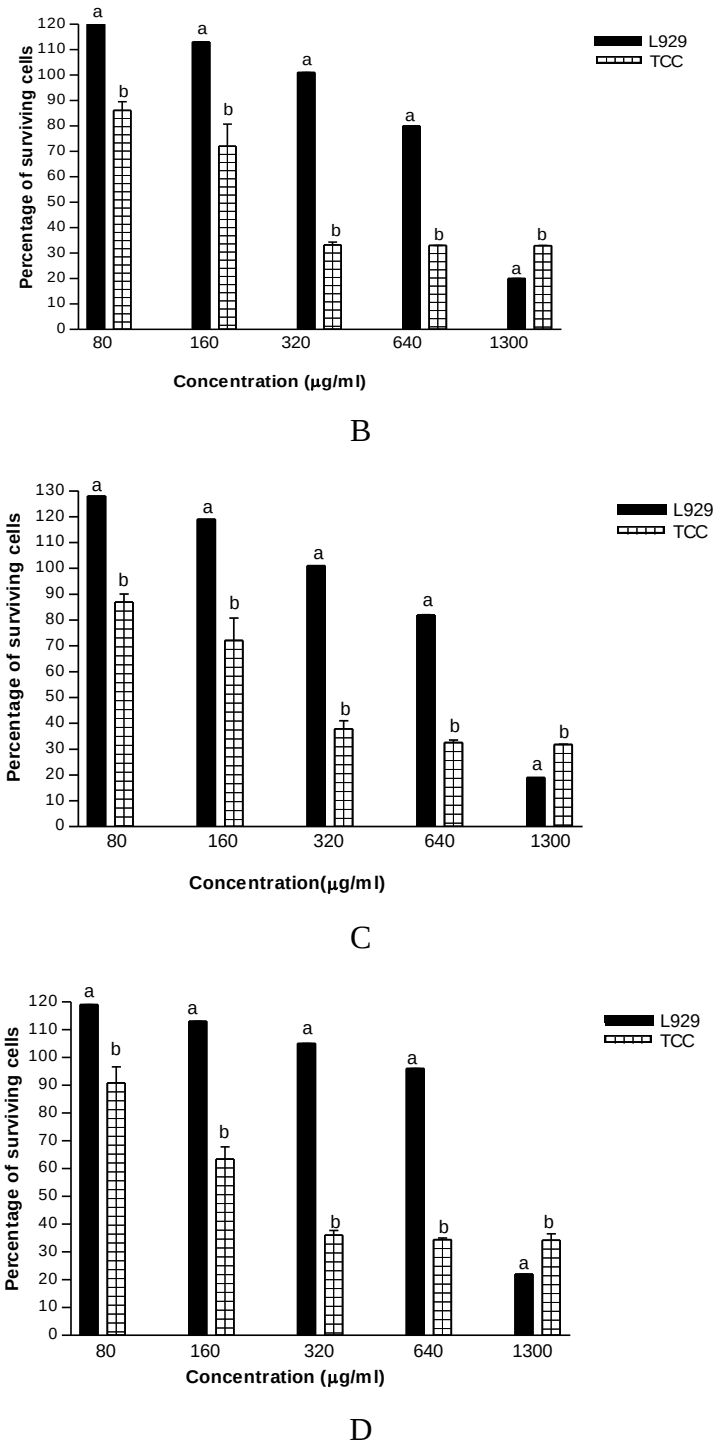

Figure 2. Surviving percentage of TCC and L929 cells at 48 h after treatment with the essential oil extracts. The extracts were obtained from different plant collections, including (A) Bajestan 2006, (B) Neyshabour 2006, (C) Bajestan 2008, and (D) Neyshabour 2008.

Discussion

Essential oils, the highly concentrated volatile, aromatic essences of plants, are of special interest for their antiproliferative effects on mammalian cells. Most essential oils contain monoterpenes, in their molecular structures. Monoterpenes are formed in the mevalonic acid pathway in plants. This is the same pathway that makes cholesterol in animals and humans. Early on, cancer researchers realized that some aspects of cholesterol metabolism are involved in cancer growth. They then discovered that plant monoterpenes could interfere with animal cholesterol synthesis, thereby

reducing cholesterol levels and tumor formation in animals (Karlsson and Alexandria, 1997). Some monoterpenes, including limonene and menthol, inhibit hepatitis 3-hydroxy 3-methylglutaryl (HMG) COA-reductase activity and reduce serum cholesterol (Gould, 1997).

Monoterpenes prevent the process of carcinogenesis at both the initiation and promotion/progression stages. In addition, monoterpenes are effective in treating early and advanced cancers. Some of monoterpenes may be chemo-preventive agents with possible cancer blocking and / or suppressing activity. Blocking monoterpenes act during the initiation phase of carcinogenesis. This prevents the interaction of chemical carcinogens with DNA, e.g., by modulating carcinogen metabolism to less toxic forms. The cancer suppressing chemo preventive activity of monoterpenes during the promotion phase of carcinogenesis may be due to inhibition of tumor cell death and/or induction of tumor cell differentiation (Gould, 1997). In addition, monoterpens stimulate apoptosis, a cellular self-destruction mechanism triggered when the DNA content is badly damaged. This safety feature is generally activated before a cell becomes cancerous. Increasing of reactive oxygen species (ROS) is another act of monoterpens, causing the death of the cancer cells (Crowell, 1999; Young, 2005). Leaf extracts of *S. leriifolia*, rich in monoterpen components, are shown here to exhibit different levels of toxicity in cancer cell line of TCC compared to the normal cells of L929.

Probably existence of α-pinen, β-pinen and eucalyptol, as chief members of the *S. leriifolia* essential oils, with about 50% of the essential oils composition, as well as other rare components and the synergic effects of these parts are the main cause of such cytotoxic effects. The profile of the rare components seems different in extracts of this plant, collected from different locations and at different times. For example, samples collected from Neyshabour on year 2008 include components such as: nopinene (0.1%), verbenon (0.09%), β-copaen (0.11%), α-calacoren (0.23%) and neryl isovalerat (17%), which do not exist in detectable amounts in samples from Bajestan. This is in line with higher cytotoxic effects of the Neyshabour extracts. Another pivotal role attributed to monoterpens is their inhibitory effects on isoprenylation. Protein isoprenylation involves the post – translational modification of certain proteins by the covalent attachment of a lipophilic farnesyl or geranyl geranyl isoprenoid group to a cystein residue at or near the carboxyl terminus and inhibition of this process alter the protein activation. Among the crucial proteins subjected to such inhibition are small G-proteins with molecular weight of 20-26 KD, such as rac, ras and rho. Such inhibitions could alter signal transduction and result in altered gene expression. The alterations in the gene expression of mammary carcinomas lead to a G1 cell cycle block, followed by apoptosis, redifferentiation, and finally complete tumor regression in which the tumor cells is replaced by normal cells (Hosseinzadeh et al., 2000; Karlsson J. and Alexandria, 1997).

Cells use the isoprenylation process to help a protein, find its proper location within the cell. In case of ras protein, if it is not in the right place, it becomes over active and can spur cancerous cell growth (Kim et al., 2001; Yoo et al., 2005). In summary, a variety of dietary monoterpenes have been shown to be effective in the chemoprevention and chemotherapy of cancer. A cytotoxic activity of essential oils from *S. leriifolia* is being considered for further investigations *in vivo*.

Acknowledgment

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Reduction of NO production in LPS-stimulated primary rat microglial cells by Bromelain

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Abstract

Microglia, the sentries of the brain, is highly implicated in neurodegeneration as in neuroprotection. Chronic microglial activation endangers neuronal survival through the release of various potentially neurotoxic mediators including Nitric Oxide (NO). Thus, negative regulators of microglial activation have been considered as potential therapeutic candidates to target neurodegeneration, such as those in Alzheimer's and Parkinson's diseases and even in chronic epileptic syndromes. Bromelain, a mixture of cysteine proteases, derived from pineapple stem, has been reported to have anti-inflammatory and immunomodulatory effects. Neonatal rat primary microglial cells were isolated from the brain according to the Floden's method. The purity of the cultures was determined by immunostaining with an OX-42 antibody which showed a purity greater than 95%.The activation profile of microglia was investigated by determining the effects of Bromelain (1, 10, 20, 30, 40 and 50 µg/ml) on the level of neurotoxin, NO, mitochondrial activity and morphological changes in treated microglia with lipopolysaccharide (LPS) (1µg/ml), as an endotoxin. Our results showed that pretreatment of primary rat microglia with bomelain (30 µg/ml), decreased the production of NO induced by LPS (1µg/ml) treatment in a dose-dependent manner, which prevented the deramification of microglia and its phagocytic morphology. Moreover, bromelain does not show cytotoxicity at any of the applied doses, suggesting that the anti-inflammatory effects of bromelain are not due to the cell death. In conclusion, Bromelain reduces the NO synthesis *in vitro* by potentially exerting its anti-inflammatory effects. Bromelain naturally found in pineapple stem, can be considered as a useful agent for neuroprotection and alleviation of symptoms in neurodegenerative diseases.

***Keywords*:** Microglia, Bromelain, CNS inflammation, NO, Neurodegeneration

Introduction

Microglia are the primary immune cells or resident macrophages which regulate inflammatory responses in the central nervous system (CNS) and often referred to as the tissue macrophage of the brain. In the unflustered brain, these cells invariably survey their environment (Hanisch and Kettenmann, 2007; Schwab and McGeer, 2008) and disturbances during neuropathological conditions, initiate reactive responses, known as 'activation', which are modelled through specific changes in their immunological and morphological phenotype to ultimately maintain brain homeostasis (Garden and Moller, 2006).

Activation of microglial cells involves proliferation, migration to the injured site, increased expression of immunomodulators, and transformation into phagocytes to access capability of scavenging effects (Dheen et al., 2007; Glezer et al., 2007). Secretion of some soluble proinflammatory molecules such as cytokines, chemokines, oxidative radicals, and nitric oxide is linked to activation of microglia (Minagar et al., 2002). The beneficial or detrimental effects of these molecules are related to their concentrations. Proinflammatory and cytotoxic factors produced by microglia have been shown to induce neurodegeneration. However, it is increasingly more appreciated that these factors may work synergistically to damage neurons (Chao et al., 1995; Jeohn et al., 1998; Skaper et al., 1996). Inflammation is now widely accepted to underlie the pathology of various neurodegenerative diseases, with evidence that the overactivation of microglia is a main participant and thus may be a target for therapeutic benefit (Block et al., 2007).

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Moreover, therapeutic intercession might be achieved by targeting primary microglia. In the healthy brain, microglia have a typical ramified morphology consisting of a small cell body and long processes with secondary branching. Changes in the microglial morphology are observed in a wide range of the CNS pathologies such as brain injury, ischemia, infection, autoimmunity, and neurodegenerative diseases (Suzumura et al., 1991). Under these pathological conditions, ramified microglia transform into amoeboid microglia, with their processes retracted and their cell body enlarged. Such a morphological transformation is associated with microglial activation and is induced *in vitro* by a variety of stimuli including LPS (Bohatschek et al., 2001; Giulian et al., 1995; Kloss et al, 2001).

LPS is an endotoxin from Gram-negative bacteria, which arouses inflammatory and immunological responses. Through binding to LPS-binding protein (LBP) in plasma, LPS is delivered to the cell surface receptor CD14. Then, LPS is transferred to the transmembrane signaling receptor toll-like receptor 4 (TLR4) and its accessory protein MD2. LPS-stimulated murine microglia, macrophages, and Kupffer cells activate the inflammatory responses and subsequently, production of inflammatory molecules (Geppert et al., 1994; Jeng et al., 2005).

Since chronic microglial activation has been implicated in the neuronal destruction associated with various neurodegenerative diseases, the activation of counter-regulatory mechanisms is essential to avoid the exacerbation of CNS inflammatory processes (McCarthy, 2006). This may be possible via the identification of agents that target overactivated microglial cells and the determination of their anti-inflammatory mechanisms.

Bromelain is an extract from pineapple stem (*Ananas comosus*) which is known for its anti-inflammatory effect (Seligman, 1962). Many Pharmacological activities of bromelain have been reported, such as regulation of immune functions, anti-inflammation, anti-edema, anti-hypertension, reduction of thrombogenesis and inhibition of cancer cell growth (Chandler and Mynott, 1998; Maurer, 2001; Taussing and Batkin, 1988). The pleiotropic therapeutic effects of bromelain are considered to be due to the complex natural mixture of closely related cysteine proteinases, proteinase inhibitors, phosphatases, glucosidases, peroxidases, and other undefined compounds (Harrach et al., 1998; Harracj et al., 1995). In addition, bromelain has demonstrated to have both antiproliferative and antimetastatic effects in tumor models *in vitro* and *in vivo* (Grabowska et al., 1997; Taussig et al., 1985). Bromelain can simultaneously enhance and inhibit immune cell responses *in vitro* and *in vivo* through a stimulatory action on accessory immune cells and a direct inhibitory action (Engwerda et al., 2001; Hou et al., 2006).

In human macrophages/monocytes and mixed lymphocyte culture, bromelain induced a significant increase in interleukin (IL)-6, tumor necrosis factor alpha (TNF-α) and interferon gamma (IFNγ) (Barth et al., 2005; Rose et al., 2005). Bromelain also enhanced IFNγ mediated TNF-α and NO production by murine macrophages. Bromelain's effect is independent of endotoxin receptor activation and is not caused by direct modulation of IFNγ receptors. Instead, bromelain either enhances or acts synergistically with IFNγ receptor-mediated signals (Engwerda et al., 2001).

On the other hand, bromelain blocks activation of extracellular signal-regulated kinase (ERK) in Th0 Cells stimulated via the T cell receptor, or stimulated with combined PMA and calcium ionophore. However, this inhibitory activity of bromelain is dependent on its proteolytic activity (Mynott et al., 1999). Bromelain also reduced LPS-induced cyclooxygenase 2 (COX2) mRNA, in bv2 microglial cells (Jeng et al., 2005). However, the efficacy of bromelain in reducing inflammation of primary microglial cell has not been reported.

Thus, we examined whether bromelain, as a natural plant extract, repress microglia activation and thereby confer neuroprotection against inflammation-related neuronal injury. So, in this study, highly enriched microglial primary cultures obtained from newborn rat cortical regions were cultured in the presence of LPS and bromelain. Activation, cytotoxicity and release of the oxidative and inflammatory factor, NO, were investigated.

Materials and Methods

Materials and reagents

Fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM) and Griess reagent were purchased from GibcoBRL (Grand Island, NY, USA). LPS (E5:055), 2', 7'-dichlorodihydrofluororescein diacetate, bromelain, E64 and Thiazolyl blue (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). FITC conjugated anti-OX42 antibody to rat CD11b/c was from CALTAG (Sketty, Swansea).

Inhibition of proteolytic activity of bromelain

Bromelain (10 mg/ml) diluted in 3 μM dithiothreitol (DTT) was incubated with 100 μM E-

64 and 60 mM sodium acetate (pH 5) for 10 min at 37°C. The inactivated bromelain was then dialyzed overnight in phosphate-buffered saline (PBS) at 4°C. The total inactivation of bromelain was achieved as assayed with the casein (Huang et al., 2008).

Cell culture

Primary microglial cells were prepared from cerebral cortices of one-day-old rat pups as described previously (Jung et al., 2003; McCarthy and De Vellis, 1980). Briefly, Cells were cultured in Dulbecco's Modified Eagle's Medium, supplemented with 100 UI/ml penicillin G, 100 µg/ml streptomycin, 2 mmol/L L-glutamine, 0.011 g/L pyruvate, and 10% fetal calf serum, seeded on polystyrene culture dishes (Nunc), and incubated in a humidified atmosphere with 5% CO2 at 37°C. To obtain primary microglia rich mixed cultures, after 2 days, all media and tissues were removed and fresh media was replaced.

Isolation of microglia

After the cells became confluent at 12–14 days (figure 1), the flasks were shaken to remove the microglia (Floden and Combs, 2007). Microglia was plated onto 96 well tissue culture plates (20,000 cells/well). The detached microglial cells were incubated for 1 h, and the non-adherent cells were removed. The adherent microglial cells were cultured for 24 h, and the purity of the cultures was routinely greater than 95% as judged by immunostaining with an anti-OX-42 antibody (figure 2).

Drug treatment

Primary microglia were pretreated with bromelain (1, 5, 10, 20, 30, 40 and 50 µg/ml) in fresh medium containing 1% fetal bovine serum (FBS) for 1 h before LPS (1 µg/ml) was added and then were incubated for 24 and 48 h.

Cell viability assay

For the cell viability assay, After various treatments, the medium was removed and the cells were incubated with MTT (3-4-5-dimethylthiazole-2-yl-2, 5-diphenyl-tetrazolium bromide) solution (1 mg/ml) in two volumes of culture medium for 4 h at 37˚C. The MTT solution was then removed, the formazan crystals in cells were dissolved in DMSO (dimethyl sulfoxide), and the level of MTT formazan was determined by measuring its absorbance at 580 nm using a microplate reader.

Nitrite assay

The production of NO was assessed as nitrite (NO_2) accumulation in the culture medium of three independent experiments 24 and 48 h after treatment, using a colorimetric test based on Griess reagent (Wilms et al., 2007) and a standard $NaNo_2$ solution was used for the standard curve.

Statistical analysis

One way ANOVA followed by the LSD test was used to determine the statistical differences among groups. Student's t-test was used to compare two groups. Values of $p<0.01$ were considered as significant, compared with the LPS-treated group without bromelain.

Results

Morphological assessment of primary rat microglia exposed to bromelain

Different morphological phenotypes of primary rat microglial cells observed with phase contrast microscope such as ramified morphology in the control isolating microglia, ameboid activated Microglial cells and intermediate phenotypes in the effective concentrations (10-30 µg/ml) of bromelain, which prevented the deramification of microglia in a dose-dependent manner (figure 3).

Bromelain decreases production of NO in LPS-stimulated primary microglia

We initially determined the effect of bromelain on NO expression in LPS-stimulated primary microglia. To analyze NO production, primary microglia were pretreated with bromelain (1, 5, 10, 20, 30, 40 and 50 µg/ml) for 1 h prior to stimulation with LPS (1 µg/ml) for 24 and 48h, the levels of NO in the culture media were determined using the Griess assay. As shown in figure 4A, LPS alone was able to markedly induce NO production from the cells as compared to that in the control. However, bromelain at 10-30 µg/ml effectively diminished the levels of NO production in LPS-stimulated primary microglia in a dose-dependent manner and a significant decrease of NO was observed only in LPS-stimulated microglial cultures exposed to 30 µg/ml of bromelain after 48 h.

Evaluation of toxicity of bromelain concentrations

To exclude the possibility that the cytotoxic action of bromelain inhibited LPS-stimulated NO production, we investigated the effect of bromelain on cell viability. Under the experimental conditions described above, there was no significant reduction

in cell viability using the MTT assay (figure 4B). Therefore, the inhibitory effect of bromelain on LPS-stimulated NO production was not due to bromelain's cytotoxic action on primary microglia.

Effects of post-treatment of bromelain on LPS-stimulated inflammatory responses

To determine if post-treatment of bromelain has neuroprotective action as well, microglia were stimulated with LPS first and then exposed to bromelain. Thus, we performed the experiment by adding bromelain 1 h after the LPS exposure. Bromelain didn't confer protective effects against LPS-induced nitrite release when exposed after the LPS stimulation. These results demonstrated that only pre- and not the post- treatment with the bromelain, suppressed the LPS-induced NO production in primary rat microglial cells.

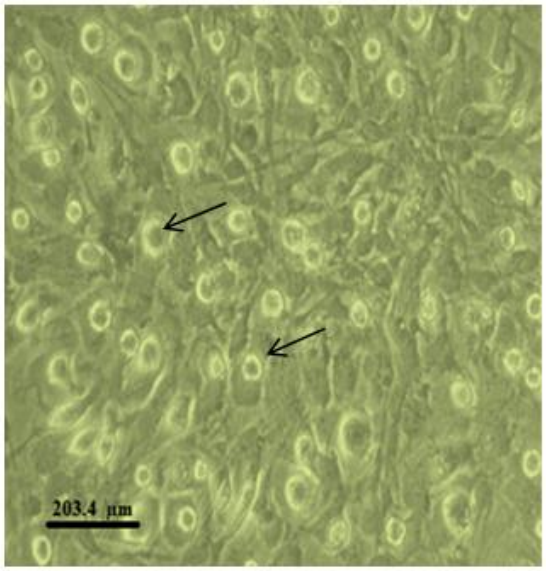


Figure 1. Confluent primary microglia-rich mixed culture at 12–14 days. Loosely adherent microglial cells (arrows) can be removed easily by shaking such a culture.

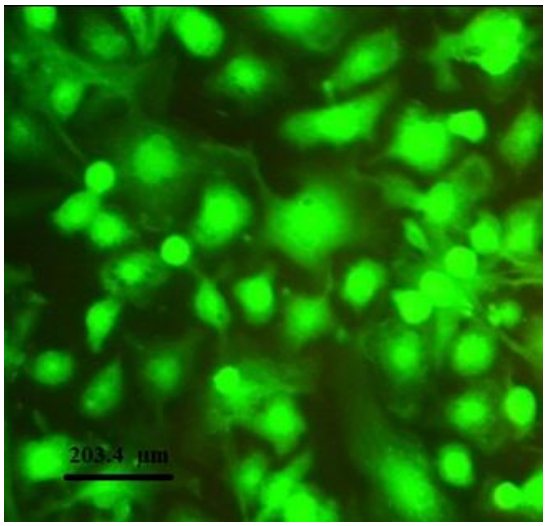


Figure 2. Isolated microglial cells immunostained with an anti-OX-42 antibody, showed more than 95% purity.

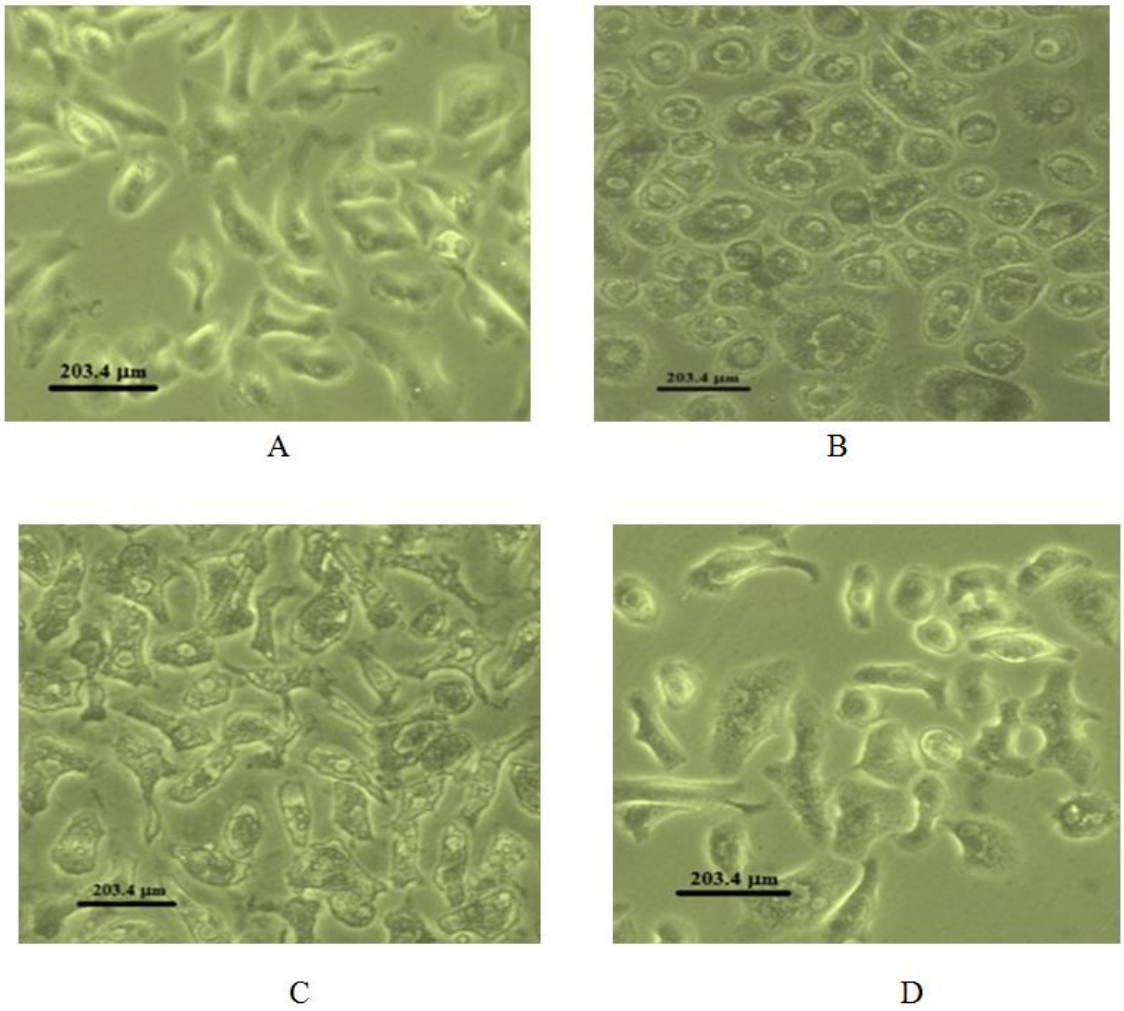
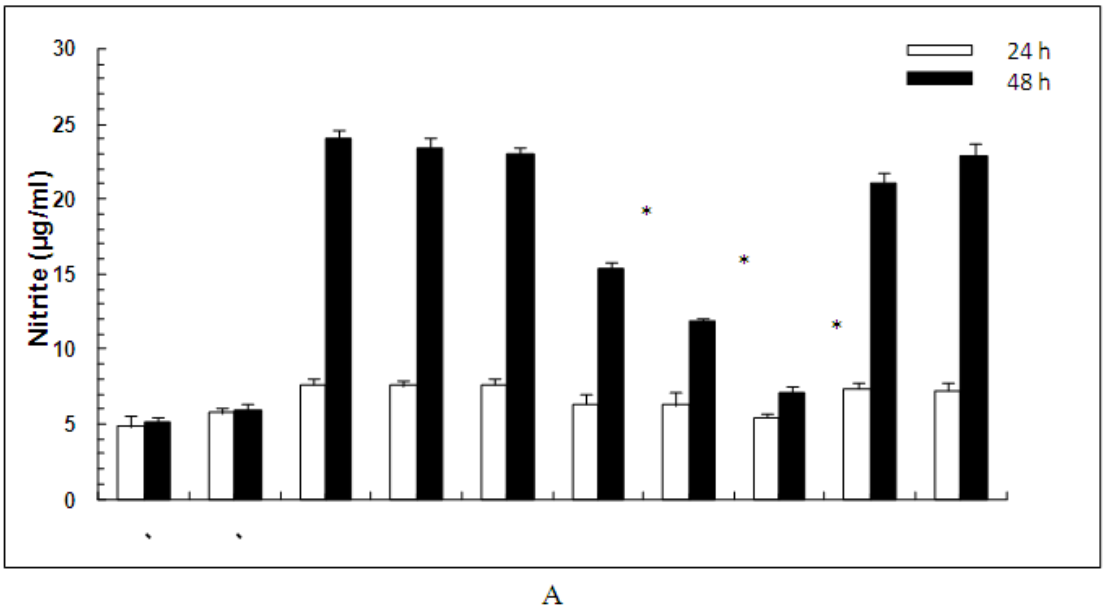


Figure 3. The effects of bromelain on microglial morphology. Isolated microglial cells were either untreated (A) or treated for 48 h with LPS 1 µg/ml (B), bomelain 10 µg/ml (C) and bromelain 30 µg/ml (D) for 1 h before LPS, and then different cellular morphology was examined under phase contrast microscope such as typical ramified morphology in control cells (A), amoeboid morphology in LPS-treatment microglia (D) and intermediate phenotypes in the effective concentrations of bromelain (B, C) which prevented the deramification of microglia in a dose-dependent manner. It should be mentioned that figures are from different fields and in fact treatments didn't have any effect on the number of the cells.



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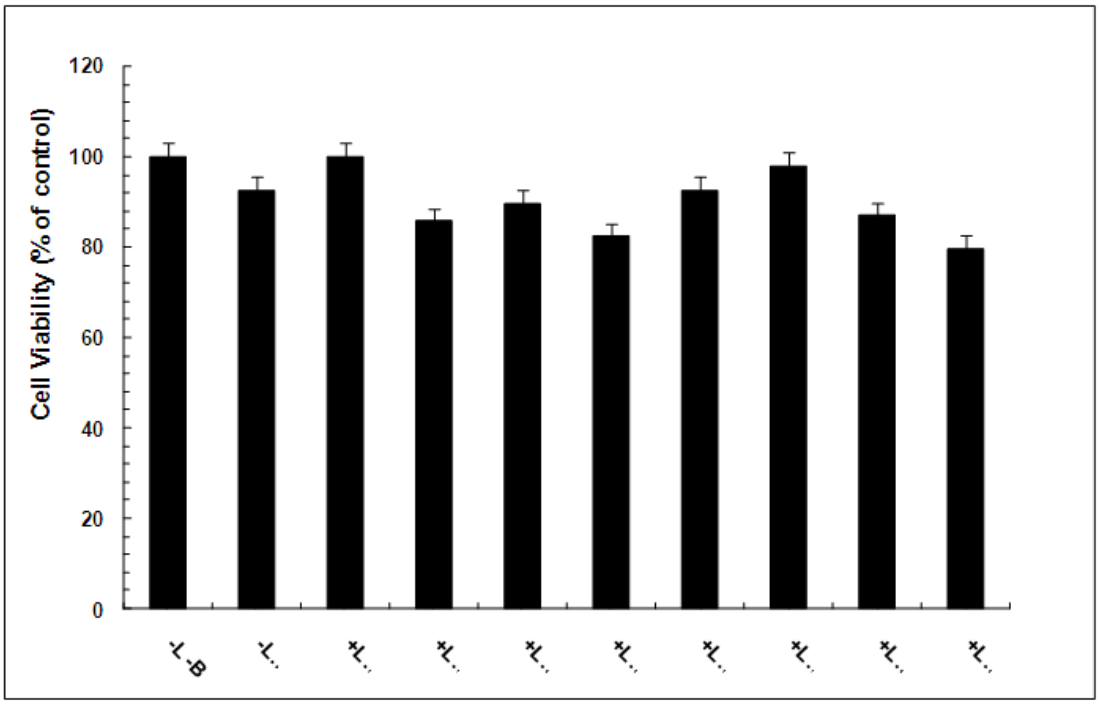

Figure 4. Effects of bromelain on NO production in LPS-stimulated microglial cells. Primary microglial cells were incubated in the absence or presence of LPS (1 µg/ml). The cells were pretreated with various amounts of bomelain (1, 5, 10, 20, 30, 40 and 50 µg/ml) for 1 h before LPS was added. The cultures were subjected to a nitrite assay after 24 and 48 h (A) and a cell viability assay after 48 h (B). *P < 0.01 as compared with the LPS-treated group without bromelain.

Discussion

Activated microglia produce various proinflammatory cytokines and free radicals such as NO, that have an important role in the process of neuroinflammatory diseases. Several lines of evidence have indicated that the NO production is upregulated in the activated microglia (Mcgeer et al., 1993). In this study, we demonstrated that bromelain, a mixture of cysteine proteases of the pineapple stem, decreased inflammatory activation of microglia in culture.

So far, contradictions exist for bromelain effects on inflammation. Bromelain has been shown to simultaneously enhance and inhibit immune cell responses *in vitro* and *in vivo* (Engwerda et al.,2001; Hou et al., 2006). Although bromelain 50 µg/ml increased the production of IFN-γ stimulated nitrite in murine macrophage cell line (Engwerda et al., 2001), elsewhere, in concentration of 100 µg/ml, it significantly inhibited the enhanced production of LPS-induced nitrite in same cell line (Wen et al., 2006). Bromelain also have anti-inflammatory effects on LPS activated microglial cell line (Hou et al., 2006).

Our results showed that bromelain (30 µg/ml) reduced the LPS-stimulated NO production in primary rat microglia cells in a dose- and time-dependent manner. Based on the previous reports and the present results, it is clear that bromelain effects differ due to the cell type and the used doses.

It is also demonstrated that only pre and not the post- treatment with the bromelain, suppressed the LPS-induced NO production in primary rat microglial cells. So, this result makes bromelain more useful for prevention than for treatment of microglia- mediated inflammation.

Microglia has been related to disease progression and pathology in several neuroinflammatory diseases such as Alzheimer's diseases, Parkinson's diseases and HIV dementia (Block et al., 2007; McCarthy, 2006). Microglia activation has both beneficial and harmful effects on neuronal injury in neurodegenerative diseases. Overactivation of microglia contribute to neurodegenerative processes through the production of various neurotoxic factors including NO (Klegeris et al., 2007).

In this study, the level of NO as a proinflammatory cytokine was evaluated in activated primary microglia treated with bromelain. NO, as well as other proinflammatory cytokines have been implicated as important mediators in the

process of inflammation (Possel et al., 2000). Microglia activation induced by CNS injury or infection is associated with neurodegeneration and the release of NO (Gonzalez-Scarano and Baltuch, 1999). Excessive production and accumulation of nitric oxide is deleterious to neurons in the inflammation-mediated neurodegenerative processes (Schmidt and Walter, 1994). Thus, it is suggested that the search for the efficient anti-inflammatory compounds that attenuate microglial activation may lead to an effective therapeutic approach against many neurodegenerative conditions.

Our results as well as previous reports showed that LPS stimulation of microglia induced a morphological change of the cells into the round shape with a loss of processes, and activates the production of NO as a proinflammatory cytokine (Nakajima et al., 2003; Suzumura et al., 1991).

We found that pretreatment of primary microglia cultures with bromelain 30 µg/ml, largely prevented the deramification of microglia.

We also showed that the potent anti-inflammatory effects of bromelain due to a decreased production of NO is dose-dependent (10-30 µg/ml). Moreover, bromelain does not show cytotoxicity at any of the applied doses, suggesting that the anti-inflammatory effects of bromelain are not due to the cell death. The effect of bromelain on reducing proinflammatory mediators such as NO suggests that bromelain is a useful therapeutic agent.

Further studies are, however, required to evaluate a neuroprotective property of bromelain in the animal models of neurodegenerative diseases, and to understand the precise molecular mechanisms of anti-inflammatory actions of the bromelain *in vitro* as well as *in vivo*.

Nevertheless, this is the first study that has demonstrated anti-inflammatory effects of bromelain in primary microglia, suggesting the neuroprotective effects of bomelain against inflammation-mediated neurodegeneration. Future works along with this line will give rise to a novel therapeutic use of the bromelain for the treatment of neurodegenerative diseases and other inflammatory disorders.

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Amino acid diversity of antigenic sites of Iranian type O foot-and-mouth disease virus

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Abstract

Foot-and-mouth disease (FMD) is a major cause of endemic outbreaks in livestock in Iran. In this study, clinical field samples of foot-and-mouth disease virus were collected from an outbreak in Khorasan Razavi Province during April and August of 2010, and subjected to indirect sandwich ELISA and RT-PCR. The viral serotype circulating during the period was confirmed to be type O. The virus was then genetically characterized for its complete P1 genomic sequences to be compared with nine corresponding nucleotide sequences of representative foot-and-mouth disease viruses (FMDVs) registered in the GenBank. The P1-coding region was 2208 nucleotides in length with 736 encoded amino acid residues. Phylogenetic analysis revealed two major lineages of A (with three additional clusters) and B. Iranian field isolate was grouped within cluster I, most closely related to Pakistani strains PAK/39/2008 and PAK/29/2008 sharing 98.37 and 98.1% amino acid identity, respectively, demonstrating the close epidemiological links between countries in the region. In contrast, our isolate showed low amino acid identity with Italian isolate of O-2-Brescia (93.48%) and Argentinean isolate of O1 Caseros (93.75%). Based on multiple sequence alignments, comparison of sequences showed that the characteristic amino acid mutations were found in the VP1, VP2 and VP3 proteins of isolated virus. This article is the first to report on the complete P1 genomic characterization of type O FMDV circulating in Iran.

***Keywords*:** foot-and-mouth disease virus, Serotype O, P1 coding region, phylogenetic analysis, Iran

Introduction

Foot-and-mouth disease (FMD) is an acute, highly contagious vesicular disease of susceptible cloven-hoofed animals, including domesticated ruminants and pigs and more than 70 wildlife species (Thomson, 1994; Alexandersen and Mowat, 2002). Although mortality is usually very low and mostly restricted to young animals (Grubman and Baxt, 2004), drastic decrease in productivity in endemically infected countries, huge economic consequences following occurrence of outbreaks in disease-free regions and restrictions on international trade in livestock and animal products cause great losses to the livestock industry worldwide (Pendell et al., 2007; Ryan et al., 2008; Carrillo et al., 2005). Consequently, FMD is classified by the Office International des Epizooties (OIE) as one of the most important infectious diseases of livestock (OIE Terrestrial Manual, 2009). Control of the disease relies on exclusion and slaughter policy, particularly for the FMD-free countries, or vaccination in the endemic areas (Cox et al., 2009).

FMD virus (FMDV), the causative agent, is a small, non-enveloped virus containing a positive-sense, single-stranded RNA genome of approximately 8.5 kb in length (figure 1), which belongs to the *Aphthovirus* genus of the family

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Picornaviridae (Cottam et al., 2009). The genome contains a single large open reading frame (ORF) encoding a polyprotein that is subsequently cleaved by virus-encoded proteases to produce the structural and non-structural proteins necessary for virus assembly and replication. The FMDV capsid precursor P1 is processed by the 3C protease to yield four structural proteins (VP1-VP4) forming viral capsid (Belsham, 2005; Fry et al., 2005). The genetic and phenotypic variability of FMDV is a consequence of high mutation rate due to the error-prone RNA polymerase lacking proofreading activity reflecting in the existence of seven antigenically distinct serotypes (Carrillo et al., 2005); four Euroasiatic serotypes: O, A, C and Asia 1, distributed throughout South America, Middle East, Asia and some parts of Africa, and three South African Territories (SAT) serotypes: SAT1, SAT2 and SAT3 (Klein, 2009). Further numerous and constantly evolving variants and lineages among the seven serotypes are described as topotypes (Grubman and Baxt, 2004). The VP1 coding sequence (less than 10% of the whole genome) is used extensively in molecular characterization, serotyping and determining evolutionary dynamics of FMDVs needed for epidemiological studies (Knowles and Samuel, 2003).

There are hundreds of FMD outbreaks in Asia, Africa, and South America each year (Sumption et al., 2008). Middle East is an FMD-endemic region of the world in which several countries have reported the epizoootics in recent years (Parlak et al., 2007; Schumann et al., 2008; Jamal et al., 2010). With the exception of SAT3, all FMDV serotypes have been isolated from susceptible populations of the Middle East, although the occurrences of types C, SAT 1 and SAT 2 have been rare (Sutmoller et al., 2003). Iran has one of the highest reported rates of FMD cases per year, and in some cases the outbreaks are major ones affecting large numbers of farms throughout the country. In recent years, types A (1996, 1999, 2005), O (2001), and Asia 1 (2004) have been the predominant types, isolated during the outbreaks (Marquardt and Freiberg, 2000; Knowles and Samuel, 2005; Knowles et al., 2009; Valarcher et al., 2009). The disease has been controlled by mass vaccination of susceptible animals, restriction of animal product and movements, quarantine and other sanitary measures, but introduction of new virus strains from neigh boring countries have caused several epidemics in the country. Therefore, regular monitoring of field outbreaks is essential for timely detection of the emergence of new strains, tracking the virus movement and implementation of any control program through vaccine strategy.

As variations in the amino acid sequence of structural proteins of FMDV are the bases for the antigenic diversity of the virus, here, the P1 genomic sequences of field isolate of type O obtained during the endemic outbreak of mid-2010 in Khorasan Razavi Province of Iran were genetically characterized, analyzed, and compared with others available on public databases.

Materials and Methods

Collection of clinical samples

During an outbreak between April and August 2010, 25 tongue epithelium tissue samples were collected from clinical FMD-suspected cattle in Khorasan Razavi Province, northeastern Iran, bordering Afghanistan and Turkmenistan countries (figure 2). By the time, infected cattle received vaccination program. Field samples were first contained in equal volumes of glycerol and 0.04 M phosphate-buffered saline (PBS) solution (OIE Terrestrial Manual, 2009) to the Central Diagnostic Laboratory of Iran Veterinary Organization of Khorasan Razavi for diagnostic confirmation. This process was performed using FMDV antigen typing ELISA (World Reference Laboratory, Pirbright, UK) genomic analysis the samples were sent to the Veterinary and Biotechnology Research Division, Razi Vaccine and Serum Research Institute, Mashhad.

RNA extraction and cDNA synthesis

Total RNA was extracted from 200 µl epithelial suspension using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. The cDNA synthesis for FMDV type O and P1 genes was carried out using P33/Rev, UKFMD/Rev7 primers, respectively (table 1) and RevertAidTM First Strand cDNA Synthesis Kit (Fermentase) in a reaction mixture containing 3 µl of RNA (330 ng/µl), 2 µl of reverse primers, and 8 µl of DEPC treated water, incubating at 65ºC for 5 min, followed by adding 4 µl of buffer 5x, 2 µl of dNTPs, 1 µl of RNase inhibitor and 1 µl of M-MuLV Reverse transcriptase. The final mixture was then incubated at 42ºC for 60 min, followed by 72ºC for 5 min. The amount of product was quantified using a NanoDrop (Technologist, USA) spectrophotometer.

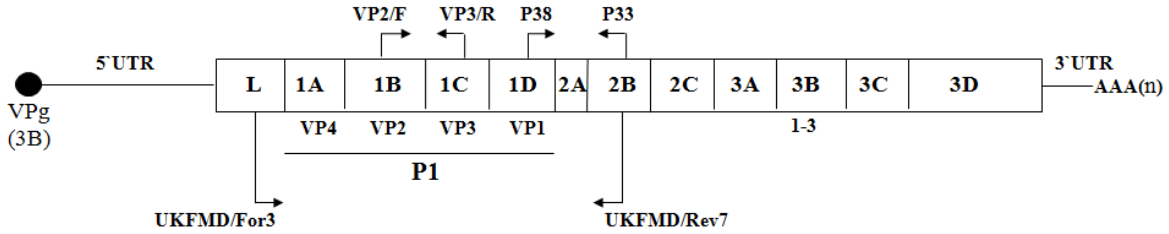

Figure 1. Schematic of the FMDV genome and three pairs of primers (table 1) used for gene amplification and sequencing. P1 represents the capsid coding region for viral proteins (VP) 1 to 4.

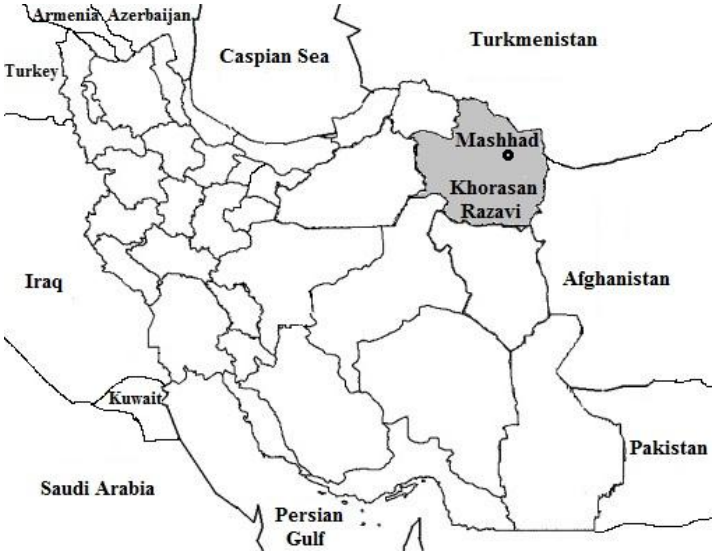

Figure 2. Map of Iran showing the geographical location of sample collection (Khorasan Razavi Province) during FMD outbreak in 2010.

Table 1. Summary of primers used for serotype detection, amplification and sequencing of P1 gene of FMDV.

Primer	Sequence (5' to 3')	Fragment (position)	Usage	Reference
P38	GCTGCCTACCTCCTTCAA	1D (3731-3748)	PCR & Sequencing	(Reid et al., 2001)
P33	AGCTTGTACCAGGGTTTGGC	2B (4113-4132)	RT, PCR & Sequencing	(Reid et al., 2001)
UKFMD/For3	CCACGCTGGCATCTTCCTGAAAG	L (1496-1518)	PCR & Sequencing	(Cottam et al., 2009)
UKFMD/Rev7	CCAGTGGCCAGTTCCTCAAATG	2B (4060-4081)	RT, PCR & Sequencing	(Cottam et al., 2009)
VP2/F	GAAACCAGTTCAACGGAGGATG	1B (2281-2302)	Sequencing	This study
VP3/R	TGGTAACCGCAGCTCGAAGT C	1C (3219-3239)	Sequencing	This study

PCR amplifications

A 402 bp fragment, including part of the VP1 coding region and its downstream sequences required for serotype O diagnosis, was amplified using GenPak®PCR MasterMix Core kit (Isogene Lab. ITD., Moscow, Russia). PCR reaction was performed in a final volume of 20 µl containing 1 µl cDNA, 2 µl (10 pmol) of each universal primers of P38 and P33 (table 1), 10 µl PCR diluents, and 5 µl DEPC treated water. The PCR conditions were as follows: 94°C for 3 min for 1 cycle; 94°C for 45 s, 57°C for 45 s, and 72°C for 45 s for 35 cycles of each, followed by a final extension of 72°C for 10 min.

A 2588 bp fragment containing the whole P1 genomic coding region was amplified using the AccuPowerTM TLA PCR PreMix kit (Bioneer). For this, TLA DNA polymerase with high fidelity and ability to amplify long fragments was used with 2 µl of each UKFMD/For3 and UKFMD/Rev7 primers (table 1), and 1 µl cDNA in a 20 µl reaction volume. The thermocycling profile was 94ºC for 3 min for 1 cycle; 94ºC for 1 min, 59ºC for 1 min, and 72ºC for 1.5 min for 35 cycles and final extension at 72ºC for 10 min. The products were then checked on 1.5% agarose gel electrophoresis together with M50 and 1 kb marker (Fermentase) for visualization.

Gene sequencing and bioinformatic analysis

The P1-amplified DNA fragment was purified using the MEGAquick-spin PCR and Agarose Gel DNA Extraction System (iNtRON Biotechnology,

INC), and subjected to sequencing directly by Comfort Read (CR) method in MWG-Biotech Pvt. Ltd. Germany. One pair of primers was designed to cover the entire P1 coding region. The primer sets used for sequencing are listed in table 1.

The resultant P1 gene sequence of Iranian FMDV serotype O were compared with nine corresponding nucleotide sequences of representative FMDVs from the GenBank database including, three Indian strains (O/India/R2/75 accession number AF204276, IND 53/79 accession number AF292107I and O/India/75Madras accession number AY145897), two Pakistani strains (PAK/29/2008 accession number GU384684 and PAK/39/2008 accession number GU384685), Turkish strain O1/Manisa/Turkey/69 accession number AJ251477, Italian strain O-2-Brescia accession number M55287, Argentinean strain O1 Caseros accession number U82271, and British strain UKG/14524 2001 accession number EU552184. Multiple alignments and pairwise comparison of nucleotide and amino acid sequences were performed by CLC Main Workbench 5.5 using the Jukes and Cantor method. The phylogenetic tree was obtained using the neighbor-joining method as executed in the computer program MEGA4. Bootstrap analysis was performed with 1000 replications on the phylogenetic tree to estimate the reproducibility of the tree topology. Complete P1 sequences of the isolate determined in this study was submitted to GenBank, with accession number HQ663879.

Results

FMD Virus isolation during the local outbreak

Collected samples examined serologically for presence of antibodies to the FMDV responsible for the outbreak, and typed as serotype O, tested with serotype-specific primers P38/P33 (table 1) by amplifying a 402 bp product. Of these, P1 gene of one sample was sequenced, which was the basis for the genetic analysis of the isolate.

Nucleotide sequence of capsid-coding region and amino acid sequence comparison

The overall length of the P1 sequence of FMDV O, isolated in the present study, covers 2208 nucleotides (nt), and the deduced amino acid (aa) sequences were 736 residues in length. Alignment of the fragment with the known sequences revealed 81 nt substitutions from which 30 (37.04%) happened in VP1, 25 (30.86%) in VP3, 23 (28.4%) in VP2 and three (3.7%) in VP4 (data not shown), compared to those of consensus sequence. Some of the nucleotide changes did not affect the aa sequence, so that only nine aa were found to be variable. The aa variations were observed in three structural proteins, VP2, VP2, and VP1, with the exception of VP4 region being well conserved (figure 3). Comparison of amino acids of antigenic sites located on P1 of Iranian isolate with those of consensus sequences showed three replacements at residues 133 (Asparagine to Aspartic acid), 140 (Serine to Arginine) and 141 (Valine to Threonine) of site I on VP1 and two changes at residues 74 (Proline to Serine) and 133 (Glutamine to serine) of site II on VP2. Substitutions in non-antigenic sites were Serine to Asparagine at residue 35 of VP3, Threonine to Alanine, Alanine to Valine, and Threonine to Serine at residues 60, 72 and 174 of VP1, respectively (figure 3).

Phylogenetic analysis

The complete P1 genomic sequences of about 2.2 kbp in length of our type O isolate, were analyzed and compared with those of overall 9 serotype O FMDV representatives deposited in the NCBI GenBank database to date. In the phylogenetic tree (figure 4), isolates were grouped in two main independent lineages A and B. Lineage A was more diversified and three additional clusters (I, II, and III) could be distinguished. The field isolate Iran/1/2010 was grouped within cluster I, most closely related to Pakistani strains of PAK/39/2008 and PAK/29/2008 and British strain of UKG/14524 2001 Accu C1, with 94.93, 92.48 and 91.98% nt identity, respectively, followed by O1/Manisa/Turkey/69 (89.04%) and Indian strains of IND 53/79 (88.77%), O/India/R2/75 (88.54%) and O/India/75Madras (87.55%). The P1-based nt comparison also revealed the least level of identity with O-2-Brescia and O1 Caseros, sharing 84.06 and 84.42% identity, respectively (figure 4). Lower aa similarity was found when comparing this Iran/1/2010 isolate to Italian isolate of O-2-Brescia and Argentinean isolate of O1 Caseros, sharing 93.48 and 93.75% aa identity. On the other hand, the present isolate was phylogenetically closest to strains PAK/39/2008 and PAK/29/2008, sharing 98.37 and 98.1% aa identity, respectively.

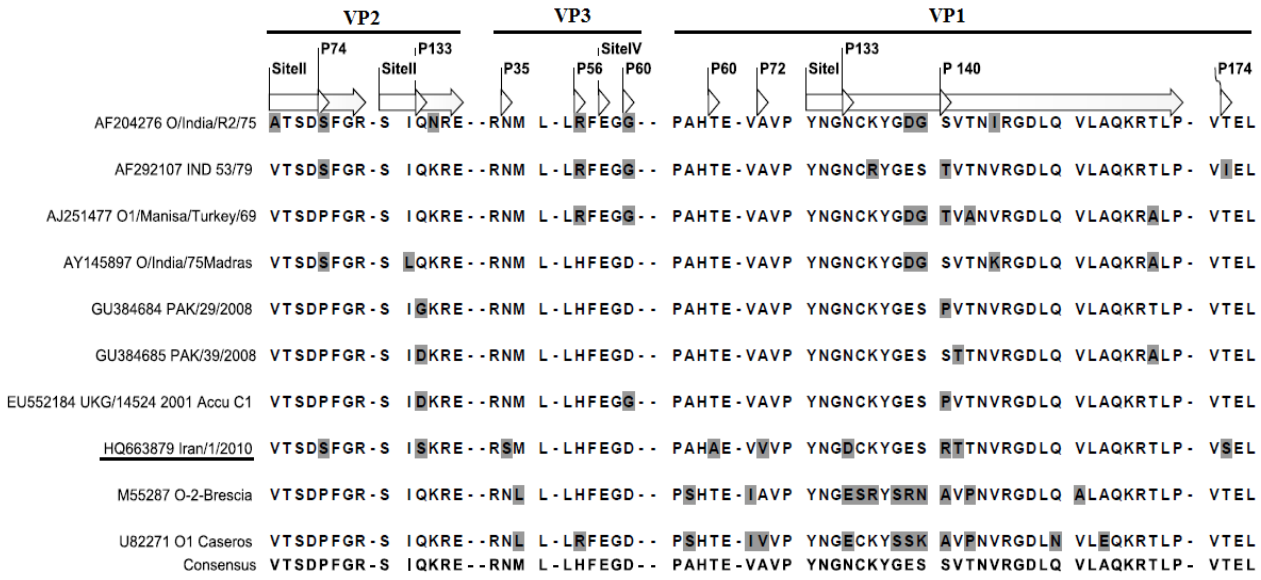

Figure 3. Alignment of the deduced amino acid sequences of P1 surface proteins (VP1-3) of type O FMDV isolate Iran/1/2010 (underlined) compared with other serotype O FMDV representatives worldwide. Arrows indicate sites with unique amino acids with numerals of their positions. Amino acids that are different from the consensus sequence are highlighted.

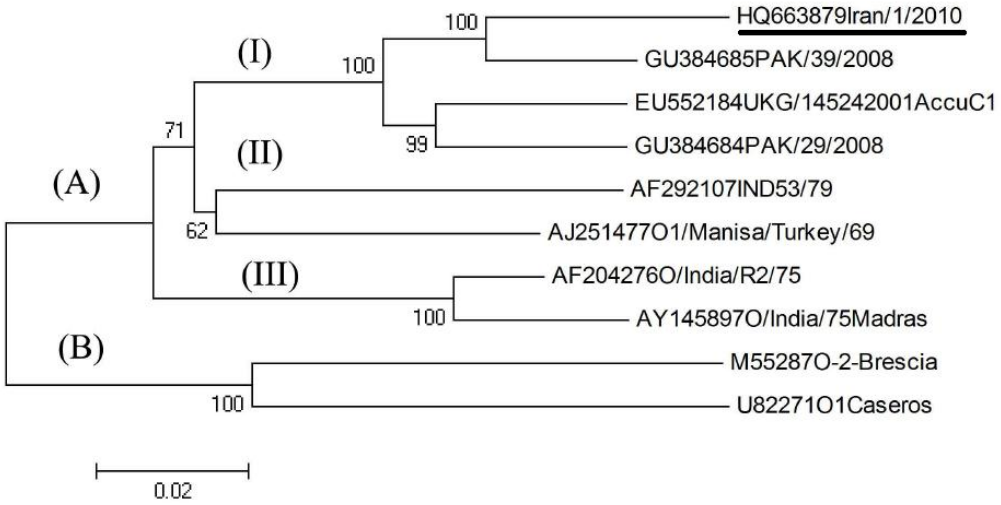

Figure 4. Phylogenetic tree based on complete P1 gene sequences showing evolutionary relationships among 10 FMDV type O representatives worldwide. The bootstrap values with 1000 replicates are indicated on the nodes. A bar represents 0.02 substitutions per nucleotide position. Iranian isolate (Iran/1/2010) sequenced in this study is underlined.

Discussion

FMDV is believed to exist as quasispecies and to be characterized by high mutation rates (10^{-3} to 10^{-5} mutations per nucleotide, per replication cycle) resulting in high genetic diversity and the evolution of new variants (Domingo et al., 2004). In Iran, the field FMD situation has been complicated by the existence of FMDV strains of multiple serotypes. Therefore, detection and sequence analysis of the pathogenic agent in case of FMD outbreaks, especially in vaccinated populations are essential to understand the epidemiology of the virus, emergence of new lineages and mutations that occur over a period of time.

The viral ORF encodes a single polyprotein, which is subsequently cleaved by viral proteases into 12 mature proteins (figure 1), including four structural proteins or P1 (1A, 1B, 1C, 1D) and eight non-structural proteins (L, 2A, 2B, 2C, 3A, 3B, 3C, 3D) (Belsham, 1993). The outer capsid proteins (VP1, VP2 and VP3) are directly involved in antigenicity and can bind to a subset of RGD-dependent integrins and heparin sulfate proteoglycan receptors on the cell surface, while VP4 is located internally. FMDV can identify cell surface integrin molecules by its RGD tri-peptide (Arginine-Glycine-Aspartic Acid) motif (Mason et

al., 2004).

Five neutralizing antigenic sites or epitopes have been reported for type O FMDV, three of which (sites 1, 3 and 5) are mapped at the VP1, and changes in their amino acid residues have been found to be associated with antigenic variability of the virus because of VP1 dual function in cell receptor binding and antigenic determination (Kweon et al., 2002). The GH loop (residues 130–160) and C-terminus (residues 200–213) of VP1 contribute to antigenic site 1, with critical amino acid residues at positions 144, 148, 154 and 208. This site is the most important epitope for FMDV. Other critical residues are 43 and 44 for antigenic site 3, and 149 for site 5. Amino acid residues at positions 31, 70–73, 75 and 77 of VP2 contribute to antigenic site 2 and position 58 of VP3 has been reported to be critical for antigenic site 4 (Kitson et al., 1990; Crowther et al., 1993; Aggarwal and Barnett, 2002; Burman et al., 2006; Carrillo et al., 2007). Among four structural proteins, the VP4, VP2 and VP3 regions are more conserved within the different FMDV strains. In addition, there is highly conserved RGDL sequence located on positions 145–148 of VP1 (Jackson et al., 2000). Considering these data, sequencing of entire P1 gene and comparison of its amino acid sequence will contribute to a better understanding of antigenic sites on the structural proteins, and may help in the development of alternative vaccines.

Sequencing results of this study showed that the G-H loop of VP1 shows the greatest changes in a sequence, while VP4 remained rather unchanged, with only three nucleotide mutations. The high conservation of the structural protein VP4 is consistent with previous reports (Du et al., 2007; Le et al., 2010) and the fact that VP4 contributes little to viral antigenicity. Comparison of deduced amino acid sequences across the P1 region with those of consensus sequence revealed a total of two substitutions in VP3 ($N_{35} \rightarrow S$) and VP1 ($T_{60} \rightarrow A$), which are unique to Iranian isolate. Among the amino acid substitutions, found at the antigenic site 1 of VP1 of Iran/1/2010, there were two changes from polar uncharged residues to a positively charged residue ($N_{133} \rightarrow D$) and a negatively charged residue ($S_{140} \rightarrow R$) (figure 3). These changes in charge and polarity may affect the conformation of the antigenic sites substantially, and thus change the antigenicity and virulence of the virus. This notion is also supported by results reported by Lin et al. (Lin et al., 2010), who observed changes in the critical residues of the VP1 viruses isolated in Taiwan in 2009.

FMD serotype O virus is predominant among the serotypes worldwide, with 10 topotypes named Middle East–South Asia (ME-SA), Europe-South America (Euro-SA), Southeast Asia (SEA), Cathay (CHY), West Africa (WA), East Africa 1 (EA-1), East Africa 2 (EA-2), East Africa 3 (EA-3), Indonesia-1 (ISA-1), and Indonesia-2 (ISA-2) (24). The ME-SA topotype has been divided into different lineages, with O-PanAsia being currently dominant in the region. This lineage was first isolated from outbreaks of FMD in northern India in 1990, and was responsible for an explosive pandemic in Asia and extended to parts of Africa and Europe from 1998 to 2001 (Knowles and Samuel., 2003). The O-PanAsia-II variant of PanAsia lineage has been further subdivided into six sub-lineages named BAL-09, YAZ-09, FAR-09, SAN-09, ANT-10 and PUN-10. During 2010, the ANT-10 sub-lineage appeared to have become the dominant type O sub-lineage in Iran, Pakistan, Afghanistan and Turkey. Other serotypes, circulating in these countries are A and Asia-1. The A-Iran-05 lineage continues to dominate, but Asia 1 has apparently not been present in Iran, Pakistan, Afghanistan and Turkey since 2004 (OIE/FAO Annual Report, 2010). An inactivated trivalent vaccine (O, A, Asia 1) is currently used in Iran as part of FMD control program.

For multiple sequence alignments, nine serotype O, referenced P1 sequences found in the GenBank database, were used for comparison. Alignment results showed that our isolate was very similar to two Pakistani strains, previously isolated in 2008, with eight (PAK/39/2008) and nine (PAK/29/2008) amino acid variations. Phylogenetic analysis of hypervariable VP1 gene of Pakistani field isolates between 2005 and 2008 showed that all type O viruses belonged to the ME-SA topotype with the majority belonging to the PanAsia-II lineage (Waheed et al., 2010). This may suggest a relatively recent common ancestor for these isolates. Similar route of virus movement has been reported by Klein et al. (Klein et al., 2006), who analyzed VP1 sequences of serotype O field isolates collected from 1998 to 2004 in Turkey. The phylogenetic analysis demonstrated that part of Turkish isolates clustered with isolates obtained from earlier outbreaks in Pakistan and Iran. Interestingly, as shown in figure 4, the present isolate was also closely related to the British type O representative UKG/14524 2001 Accu C1, with 10 aa variations. This finding confirms the spread of O-PanAsia strain towards the west into European countries (Knowles et al., 2001). Additionally, close relation of cluster I strains to clusters II and III consisting of viruses from Turkey and India indicates their common origin.

In conclusion, this study is the first to report on

the genetic characterization of complete P1 gene of FMDV type O in Iran, and the obtained results have important implications in understanding the molecular epidemiology of circulating strains in the country, which will provide valuable information for the implementation of an effective control program. Nevertheless, it remains unknown whether the virus isolated in 2010 in Khorasan Razavi Province was evolving under evolutionary forces such as selection and genetic drift operating on populations of the virus. Because contemporary FMD outbreaks were reported in Afghanistan and Pakistan (OIE/FAO Annual Report, 2010), it is likely that the virus may have moved to Iran from neighboring countries. Therefore, further in-depth analysis of evolutionary relationships amongst contemporary viruses in the Middle East should be undertaken by reference laboratories to trace the origin of outbreak viruses. Sheep and goats play a major role in the spread of the disease, as symptoms of FMD in these species are frequently mild or unapparent (Kitching et al., 2002). However, in the outbreak occurred in 2010 in Khorasan Razavi Province, small ruminants showed obvious signs of clinical disease with unprecedented mortality. Accordingly, mandatory vaccination coverage of small ruminants at least in borderlines may minimize the severity of infection and limit the spread of disease. Further research may also help to elucidate how new viruses with altered pathogenesis and host range emerge. In addition, the study highlights the need for active surveillance of FMD, strengthening of animal movement regulations across borders, and developing a regional FMD control strategy.

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The role of Gibberellic acid on some physiological responses of transgenic tobacco (*Nicotiana tabacum* L.) plant carrying Ri T-DNA

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Abstract

Transgenic and non transgenic *Nicotiana tabacum* L (cultivar Wisconsin) containing Ri T-DNA were treated with 0, 0.2 and 0.4 mgL^{-1} GA_3 in Murashig and Skoog medium. Some physiological parameters including shoot length, leaf area, number of auxiliary bud, fresh and dry weight, number and length of trichomes were measured. Shoot length, fresh weight and dry weight were increased but number of trichome did not change by GA_3 treatment. Chlorophyll, carotenoid and anthocyanin pigments of leaf were decreased. Auxin and gibberellic acid content of leaf and root were also measured. Exogenous GA_3 increased root auxin in the transgenic plants while it did not change in shoot. GA_3 treatment increased gibberellin content in both of root and shoot.

***Keywords*:** auxin, gibberellic acid, growth parameters, *Nicotiana tabacum*

Introduction

Tissue culture provides a useful system to investigate plant hormone responses and their growth and developmental processes (Zhang et al., 2008). Gibberellins (GAs) constitute a group of tetracyclic diterpenes that best known for their influence on leaf expansion, stem elongation, flower, fruit development and plant morphology (Yamaguchi, 2008; Chauhan et al., 2010). To date, 136 GAs from higher plants (128 species) have been identified (MacMillan, 2002). GA_3 is the first widely available active form of commercial gibberellins which is economically an important secondary metabolite (Martin, 1983).

GA_s promote cell elongation by induction of enzymes involved in cell wall loosening and expansion, such as xyloglucan endotransglycosylase (XET), expansin and pectic methylesterase (PME). Several studies on different plant species have shown that the exogenous application of GA_3 can enhance the productivity of crops affecting the vital physiological process (Rahman et al., 2004; Bora and Sarma, 2006). The vegetative growth characteristics of Gladiolus and *Zantideschia aethiopica* plants were improved as a result of GA_3 treatment (Kirad et al., 2001; Prasad et al., 2002; Brooking and Cohen, 2002). GA_3 increases shoot length by increasing its rate of elongation in majority of plants, including *Brassica campestris* (Pressman and Shaked, 1991). Root length was also observed to increase by GA_3 treatment in *Lupinus albus* (Sidiras and Karsioti, 1996). GA_3 increased dry matter and leaf-area index in mustard plant (Khan, 1996), and photosynthetic rate in leaves of bean (Khan et al., 2002).

Nicotiana tabacum is a model system for tissue culture and plant science investigations (Lang, 1989) as well as useful tool for genetic transformation and expression studies (Bate and Twell, 1998; Holmberg et al., 1997). In this study, transgenic tobacco plants carrying Ri T-DNA containing auxin biosynthesis genes (AUX1 and AUX2 genes) was used (Zamanzadeh and Ehsanpour, 2011). Based on the data released by Zamanzadeh and Ehsanpour, (2011), transgenic tobacco carrying Ri-TDNA has been reported to have short shoot length with compacted auxiliary buds. The reason for this change of growth pattern and morphology of this transgenic plant has not been well understood. It is speculated that might be due to gibberellic acid and auxin interaction. Based on these data, the objectives of the present study was to understand some physiological responses of transgenic tobacco plants after treatment with gibberellic acid (GA3).

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Materials and Methods

In this study *in vitro* propagated of transgenic (T) plants originated from T1 seeds and non transgenic (NT) tobacco plants (cultivar Wisconsin) were grown in MS (Murashige and Skoog, 1962) medium supplemented with 0, 0.2 and 0.4 mgL^{-1} GA_3. After four weeks, their growth parameters including shoot length, leaf area, number of auxiliary buds, fresh and dry weight, number and length of trichomes were measured. Chlorophyll was also extracted from the leaves and measured based on method of Arnon (1949). Carotenoid content was estimated using Kirk and Allen method (1965) and illustrated as milligrams per gram fresh weight. Anthocyanin was extracted and estimated by the method of Laby (2000). The amount of IAA produced in the root and leaves were determined by the method described by Mandal et al. (2007) and Gibberellin contents was extracted and measured by Berríos (2004) method.

Results

Growth parameters

GA_3 treatments in concentrations of 0.2 and 0.4 mgL^{-1} significantly increased the length of shoots in both transgenic and non transgenic tobacco plants when compared to untreated plants, but both concentrations of GA_3 had similar effect on length of shoots. Length of shoots in the transgenic plants was significantly shorter than those of untreated non transgenic plants (table 1)

The fresh and dry weight of the non transgenic plants were increased with GA_3 treatment significantly. The fresh weight in the transgenic plants decreased, while the dry weight did not change.

The number of auxiliary buds was not affected in the non transgenic plants by GA_3 treatment, while it was significantly increased in the transgenic plants. GA_3 treatments significantly decreased the leaf area in both T and NT plants when compared to the untreated plants. Concentration of 0.2 and 0.4 mgL-1 of GA_3 had similar effect on leaf area in the transgenic plants. In non transgenic plants, the minimum value of leaf area was obtained in plant treated with 0.2 mgL^{-1} GA3. The number and the length of leaf trichomes in transgenic plants were significantly higher than those in the non transgenic plants.

Photosynthetic and non photosynthetic pigments

The effects of GA_3 on photosynthetic and non photosynthetic pigments of tobacco leaves are illustrated in table 2. GA_3 treatments with 0.2 and 0.4 mgL^{-1} equally decreased chlorophyll a, b and total chlorophyll in transgenic and non transgenic plants. Carotenoid content in the non transgenic plants increased significantly with increasing of GA_3 concentration, but decreased in the transgenic plants, especially in concentration of 0.2 mgL^{-1} GA_3. While anthocyanin content was not affected by treatment with GA_3 at 0.2 mgL^{-1} but it was significantly decreased at 0.4 mgL^{-1} of GA3 in the NT plants. The anthocyanin content of transgenic plants was decreased with increasing of GA_3 concentration too.

Table 1. Effects of GA_3 on growth parameters of tobacco (NT=non transgenic, T=transgenic). Similar letters represent no significant differences ($P<0.05$).

plant	NT	NT	NT	T	T	T
GA_3 (mg/L)	0	0.2	0.4	0	0.2	0.4
Shoot Length (cm)	28.7 ± 2.51b	75 ± 5.56a	80.7 ± 7.09a	24 ± 1.73c	66.7 ±5.68a	73.4 ± 8.62a
Shoot Fresh weight (g)	4.1 ±0.25a	4.6 ±0.7a	5.76 ±0.703b	3.68 ±0.107b	2.39 ± 0.215bc	2.2 ± 0.58c
Shoot Dry weight (g)	0.16 ± 0.019a	0.24 ±0.009b	0.28 ± .035b	0.16 ± 0.02a	0.13 ± 0.022 a	0.13 ± 0.02a
Auxiliary bud Number	0.67 ± 0.57b	0.67 ± 0.57b	0.67 ± 0.57b	1 ± 0.81b	2 ± 1a	2.67 ± 1.15a
Leaf Area (mm^2/plant)	3996.7± 255.21 a	2603.4 ± 200.4 c	3085 ± 235.16 b	3091.4±259.48 b	1468.4 ± 85.78 d	1455 ± 217.02 d
Trichome Number/mm^2	49.4 ± 5.99b	47.7 ±6.19b	50.8 ±4.25b	154.7 ± 13.29 a	155.7 ± 10.74 a	158.3 ± 7.53 a
Trichome Length (mm)	0.1437± 0.014 b	0.142 ± 0.022 b	0.1433 ± 0.016 b	0.3156 ± 0.023 a	0.309 ± 0.024 a	0.32 ± 0.029 a

Table 2. Effect of GA_3 on photosynthetic and non photosynthetic pigments of tobacco leaves (NT= non transgenic, T= transgenic).
Similar letters represent no significant differences (P<0.05).

Plant	NT	NT	NT	T	T	T
GA_3(mg/L)	0	0.2	0.4	0	0.2	0.4
Chla(mg/g FW)	1.23±0.117	0.99±0.74ab	0.86±0.09b	1.17±0.128a	0.53±0.119c	0.48±0.031c
Chlb(mg/g FW)	0.47±0.366a	0.30±0.067b	0.29±0.029b	0.43±0.053a	0.20±0.008c	0.19±0.001c
Total(mg/g FW)	1.69±0.135a	1.29±0.141b	1.16±0.119b	1.60±0.188a	0.73±0.124c	0.68±0.029c
Carotenoid (mg/g FW)	3.15±0.113d	3.50±0.060c	4.65±0.098a	3.85±0.068b	1.64±0.101f	2.08±0.106e
Anthocyanin (µg/g)	4.05±0.055a	4.14±0.134a	2.55±0.087d	4.20±0.086a	3.81±0.099b	3.33±0.088c

Auxin content

Effect of GA_3 treatments on levels of shoot and root auxin is shown in figure1. Auxin level in shoots was similar in both untreated transgenic and non transgenic plants. However, auxin level in shoots was not affected by GA_3. Data recorded on auxin level of roots revealed that GA_3 enhanced the auxin content in both plants. Auxin content of root in the transgenic plants was significantly higher than that of non transgenic plants.

Gibberellin content

Gibberellin content of roots and shoots in NT plants were significantly higher than those of transgenic plants. GA_3 at 0.2 and 0.4 mg/L showed similar effect by increasing of gibberellin content in shoots of the transgenic plant. GA_3 treatments increased the gibberellin content of the roots in both transgenic and non transgenic plants, in particular at 0.4 mg/L^{-1} concentration (figure 2).

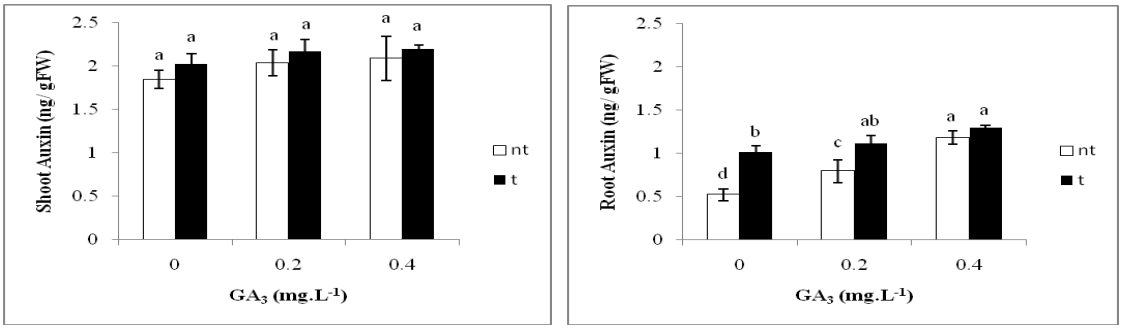

Figure 1. Effect of GA_3 on auxin content of root and shoot in tobacco plants (nt= non transgenic, t= transgenic). Similar letters represent no significant differences (P<0.05).

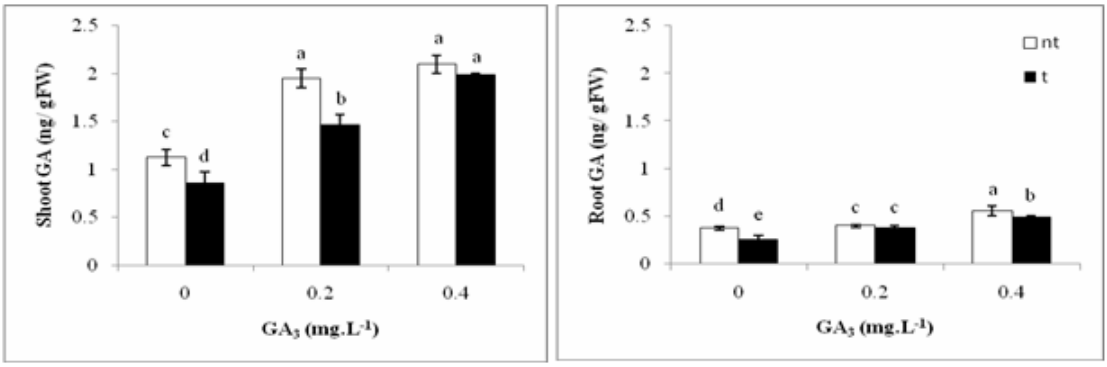

Figure 2. Effect of GA_3 on Gibberellin content of root and shoot in tobacco plant (nt= non transgenic, t= transgenic). Similar letters represent no significant differences (P<0.05).

Discussion

GA is a phytohormone which affects plant morphology as well as its physiological responses (Chauhan et al., 2010). In this study two different concentrations of GA_3 (0.2 and 0.4 mg/L) showed a dramatic effect on plant length. The plant growth consists of two steps, cell divisions and subsequent cell elongation. GA_3 has been reported to increase cell wall extensibility leading to elongation (Rahman et al., 2004). It also activates cell division in the intercalary meristem, assisting in the change of rosette plants in long stem (Iqbal et al., 2011). The increase in plant height due to GA_3 application might be due to its effect on elongation of internodes. Hully and Phillips (1995) suggested that GA_3 can increase the cell number and size by a subsequence affect on plant growth. Application of gibberellin could also promote shoot elongation. Similar observation was also reported by Xu et al. (1997). Freedborg et al. (2001) reported that exogenous application of GA_3 leads to elongation of shoots. This data supports our finding in the transgenic and non transgenic plants after treatment with gibberellin.

Plant growth regulators are chemicals with influence on plant growth when they are applied in very little quantities. It is known that the developmental processes in plants are regulated by the action and balance of the different group of growth regulators, which may act as activators or inhibitors of the metabolic processes (Ortuno et al., 1999). The increasing of fresh and dry weight in non transgenic plants may be due to an increase of protein and carbohydrate contents are reported by others (Gehan et al., 2011). Similar response was observed in our experiments when tobacco plants were exposed to GA3. Decreasing of fresh weight in the transgenic plants can be explained by some internal hormonal disequilibrium. The action of these substances depends on environmental conditions and plant characteristics and genetic potential (Vieira and Almeida, 2010).

Zhang et al. (2008) showed that gibberellin and its signaling pathway inhibit shoot bud regeneration of *Arabidopsis*. Bora and Sarma (2006) reported that in pea, GA_3 up to 250 µg/mL, was highly stimulatory on number of branches per plant, and its effect declined at higher concentrations. Based on present data, GA_3 application enhanced the auxiliary buds only in the transgenic plants. This finding might be linked to the genetic potential of the transgenic plants that is different from that in the non transgenic tobacco plants. Decreasing the leaf area in both transgenic and non transgenic plants, tested with GA_3 compared with the untreated plants, can be due to the fact that GA_3 stimulates the growth by increasing cell size and division (Jupe et al., 1988).

In our experiments when GA3 was applied to the culture medium, the number of trichomes did not change either in transgenic or non trangenic plants. This was against the report indicating that, in GA-deficient mutant (ga1-3), GA-response mutant (spy-5), and uniconazol treated tobacco plants (a GA-biosynthesis inhibitor), the trichome numbers were reduced (XueYing et al., 2007). The difference might be due to the difference between plant genotypes or experimental conditions, such as concentration of GA3 used (Bekheta et al., 2008). Application of GA_3 resulted in a decrease in the chlorophyll content in both plants in the present study. Similar results were observed in pea. It was suggested that the increase in cell volume, caused by GA_3, was not correlated with an increase in synthesis of chlorophyll content. It might however be due to dilution of the chlorophyll content in the leaves (Bora and Sarma, 2006).

Exogenous GA_3 has a negative feedback regulation effect on gibberellin biosynthesis pathway (Hedden and Phillips, 2000). Exogenous GA_3 treatment might cause the geranylgeranyl pyrophosphate precursor to enter into carotenoid synthesis pathway and increase the carotenoid content in the non transgenic tobacco plants. The result of carotenoid content of non transgenic plant is supported by findings of Munjal and Guswami (1995), the fact that when Paclobutrazol (a GA-biosynthesis inhibitor) was applied to potato the carotenoids in the leaf were increased (Tekalign et al., 2005). Decreasing of carotenoid content in the transgenic plants in the present study, shows that different responses of these plants to GA_3 might be due to existence of Ri T-DNA in these plants.

Anthocyanins are secondary metabolites, which play an important role in the physiology of plants. GAs, jasmonate and ABA, but not 2,4-D, ethylene and cytokinins, may interact or crosstalk with sucrose to form a complex web of overlapping signaling pathways that coordinate anthocyanin accumulation.

Yang et al. (1996), suggested that both auxin and GA, are indispensable factors for normal stem elongation in intact peas. GA and IAA appear to have different roles in cooperatively promoting the stem growth, with GA largely conferring increased elongation potential, principally by stimulating the cell division, and with auxin leading to the promotion of cell elongation. Our results revealed that GA_3 induced of auxin biosynthesis in the roots of both T and TN tobacco plants. Also, Law (1987)

showed that in *Pisum sativum* L. the process of elongation happened in the presence of indole-3-acetic acid and its precursors, except for L-tryptophan, which required the addition of gibberellin, for induction of growth. It is proposed that gibberellin increases the biosynthesis of indole-3-acetic acid by regulating the conversion of L-tryptophan to D-tryptophan, which is then converted to the auxin. Furthermore, Li et al. (2003) indicated that GA_3, during flower-bud induction, significantly inhibited the activities of PAL, PPO, POD and IAA-oxidase, delaying the biosynthesis of lignin and raising the level of IAA in leaves of current shoots. Treatment with 0.2 and 0.4 mgL^{-1} of GA_3 could not change significantly the IAA content of tobacco shoots, because the effects of this hormone on plants vary depending on the plant organ. More production of auxin in roots of the transgenic plants might be a response of auxin biosynthesis in the transgenic plants (Zamanzadeh and Ehsanpour, 2011).

GA_3 treatment increased the gibberellin content in roots and shoots in the both tested plants. When the grapevine was treated with GAs, a substantial increase in the GA content in the apical bud and tendril was observed (Yao et al., 2010). Exogenous application of GA_3 on *Paris polyphylla* was also reported (Li et al., 2010).

In conclusion, our data revealed that GA_3 treatment resulted in morphological change of the transgenic and non transgenic tobacco plants and alter the pigment and hormone contents. These changes under the influence of growth regulators might be due to activation of mechanisms related to the GA and IAA action as a consequence of Ri T-DND transformation.

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Chromosome number reports in five *Onobrychis* species (*O.* sect. *Onobrychis*, Fabaceae) in Iran

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Abstract

In this study the original mitotic chromosome counts are presented for 5 *Onobrychis* species of *O.* sect. *Onobrychis* in Iran, 2*n* = 2*x* = 14 for *O. persica*, 2*n* = 4*x* = 28 for *O. viciifolia*, 2*n* = 4*x* = 28 for *O. altissima*, 2*n* = 2*x* = 14 for *O. shahpurensis* and 2*n* = 2*x* = 14 for *O. sosnovskyi*. The basic chromosome numbers of all studied taxa are consistent with the proposed base number of *x* = 7. In addition, the meiotic chromosome number of 2*n* = 4*x* = 28 for *O. viciifolia* and *O. altissima* and of 2*n* = 2*x* = 14 for *O. sosnovskyi* and *O. persica* are reported here. This study is the first report on the chromosome counts of *O. persica* and *O. shahpurensis*. All studied taxa displayed regular bivalent pairing and chromosome segregation at meiosis. However, some abnormalities were observed in the taxa are discussed.

***Keywords*:** chromosome number, Fabaceae, meiotic behavior, mitosis, *Onobrychis*, Iran

Introduction

Onobrychis Miller comprises of about 170 species under 12 higher taxa mainly distributed in southwest Asia, the Mediterranean region, temperate Europe and Asia, a few of which are cultivated as fodder or as ornamentals (Lock and Simpson, 1991; Yakovlev et al., 1996; Mabberley, 1997). Boissier (1872) subdivided the genus *Onobrychis* into two sections, *Euonobrychis* Bunge and *Sisyrosema* Bunge, based on characters of indumentums and corolla. He accepted 24 species within these 2 sections. In 'Flora Iranica' (Rechinger, 1984) 54 species from Iran were treated under 8 sections, *Dendrobrychis*, *Lophobrychis*, *Onobrychis*, *Laxiflorae*, *Anthyllium*, *Afghanicae*, *Heliobrychis* and *Hymenobrychis*. The taxonomy of the genus continues to be subject of much confusion, mainly because of the different approaches to species delimitation, resulting in varying numbers of recognized species (Boissier, 1872; Sirjaev, 1925; Hedge, 1970; Ball, 1978; Duman and Vural, 1990; Aktoklu, 2001). Recently, some new taxa of the genus have been described from Iran (Ranjbar et al., 2004, 2007; Ranjbar, 2009; Ranjbar et al., 2009a, 2009b, 2010a, 2010b, 2010c, 2010d, 2010e, 2010f, 2011).

Most of the cytological studies in the genus have concentrated on the chromosome count (Baltisberger, 1991; Karshibaev, 1992; Slavivk et al., 1993), with little work focused on detailed karyological criteria for taxonomic purposes (Khatoon et al., 1991; Mesicek and Sojak, 1992). From these reports, it is evident that the chromosome count is known for just over a quarter of the species. Two basic chromosome numbers (x = 7 and *x* = 8) and 4 ploidy levels (2*n* = 2*x* = 14, 2*n* = 4*x* = 28, 2*n* = 8*x* =56 and 2n = 2*x* = 16, 2*n* = 4*x* = 32) are present in the genus (Abou-el-Enain, 2002). The elucidation of the origins of species has been greatly aided in recent years by the ability to make comparisons between putative progenitor species and their derivatives at the molecular level (Crawford, 1990; Avise, 1994). We describe here mitotic chromosome number, and meiotic chromosome number and behavior of 5 *Onobrychis* species of *O.* sect. *Onobrychis* in Iran.

Materials and Methods

For mitosis, 5 *Onobrychis* species, *O. persica*, *O. viciifolia*, *O. altissima*, *O. shahpurensis* and *O. sosnovskyi*, were collected from different locations in Iran (figure 1) and pods were collected from healthy plants. Voucher specimens were deposited at the Herbarium of the Bu-Ali Sina University (BASU), Hamedan, Iran. Then, pods were left to dry at room temperature, and seeds obtained from dry pods and kept at 4 °C until used. Young root

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tips were obtained from seeds germinated in petri dishes pretreated with 0.05% colchicine for 3 h and fixed in 3:1 ethanol: glacial acetic acid for 24 h. Root tips were hydrolyzed for 6 min in 1M HCl at 60 °C, washed briefly in dd H_2O and stained in Feulgen's solution for 1-2 h. All permanent slides were made using Venetian turpentine (Wilson, 1945). The slides were observed under an Olympus BX-41 photomicroscope.

Also the chromosome number and meiotic behavior were analyzed in all above mentioned species. 15 flower buds from at least 5 plants at an appropriate stage of development were fixed in Piennr's fluid containing ethanol (96%), chloroform and propionic acid, 6:3:2 (v/v/v), for 24 h at room temperature and then stored in 70% alcohol at 4 °C until used. Anthers were squashed and stained with 2% acetocarmine. All permanent slides were made using Venetian turpentine (Wilson, 1945). Photographs of chromosomes were taken by Olympus BX-41 photomicroscopes at initial magnification of 1000X. Chromosome counts were made from well-spread metaphases in intact cells, by direct observation and from photomicrographs.

Results

Mitotic chromosome number and ploidy level

Results from the present study showed that *O. viciifolia* and *O. altissima* are tetraploid with the base number of $2n = 4x = 28$ (figures 2A-2F), while *O. shahpurensis*, *O. persica* and *O. sosnovskyi* are diploid with the base number of $2n = 2x = 14$ (figures 2G-2T).

Onobrychis altissima Grossh. in Sc. Papers Applied Sect. Tiflis Bot. Gard. Pt. V. 141 (1929). Iran: East Azerbaijan, 10 km after Varzaghan, Mirzaali Kandi, 2010 m, Ranjbar and Hadadi 14209. Perennial herbs, stems erect or erect-ascending, 50-90 cm tall, corolla pink, 10-13 mm long, dark standard dark and wings short. It is closely related to *O. viciifolia* growing throughout Iran especially in the shape of leaf, stem indumentum, number of leaflets and flowers and also in the length of standard, keel and pod. *O. altissima* is tetraploid with the base number of $2n = 4x = 28$.

Onobrychis viciifolia Scop. Fl. Carniol., ed. 2. 2:76 (1772).
Iran: West Azerbaijan, Orumieh to Oshnavieh, after Sangar, 1650 m, Ranjbar and Hadadi 14214.
Perennial herbs, stems erect or erect-ascending, 50-90 cm tall, corolla pink, 10-13 mm long, standard dark and wings short. *O. viciifolia* is tetraploid with the base number of $2n = 4x = 28$.

Onobrychis sosnovskyi Grossh. in Sc. Papers Applied Sect. Tiflis Bot. Gard. Pt. V. 162 (1926). Iran: East Azerbaijan, Marand and Kharvanak, 1215-1725 m, Ranjbar and Hadadi 14208 and 14212. Perennial plant, (20) 40-60 cm height; stem erect, branched; corolla pink, 9-11 mm long. This species occurs in Turkey, Iran and Caucasus. It is the only long wing species in northwest Iran. This diploid species shows the basic chromosome number of $2n = 2x = 14$ (figures 2G-2J).

Onobrychis persica Sirj. and Rech. f. Repert. Spec. Nov. Regni Veg. 50: 257 (1941).
Iran: Zanjan, Zanjan to Gheidar, 1950 m, Ranjbar and Hadadi 14197. Perennial plant, 20-50 cm height; corolla pink, 8-9 mm long. It is one of the short wing species in the west of Iran that is diploid and shows the basic chromosome number of $2n = 2x = 14$ (figures 2O-2S).

Onobrychis shahpurensis Rech. f. Fl. Iranica [Rechinger] 157: 414 (1984).
Perennial plant, 25-40 cm tall; corolla white, 8.5-9.5 mm long. It is the only short wing species with white flowers in the Flora Iranica grows in west Iran. It is a diploid species with $2n = 2x = 14$ chromosome number (figures 2K-2N).

Meiotic behavior and abnormalities

Chromosome number and meiotic behavior were determined in 16 populations of 5 species. A summary of their cytological features is given in table 2, and the chromosomes are illustrated in figures 3–7. A total of 5130 diakinesis/metaphase I (D/MI), 2395 anaphase I/telophase I (AI/TI), 1350 methaphase II (MII) and 7263 anaphase II/telophase II (AII/MII) cells were analyzed. The meiotic irregularities observed in the *Onobrychis* species studied here included the occurrence of varied degrees of sticky chromosomes, formation of laggards and bridge in anaphase I & II, telophase I and II, cytomixis, cytoplasmic connections, desynapsis in metaphase I and asynchronous nuclei in metaphase II (figures 3-7).

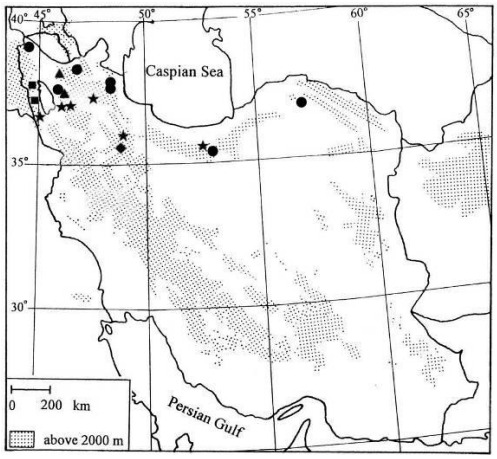

Figure 1. Distribution of *O. viciifolia* (★), *O. altissima* (●), *O. sosnovskyi* (▲), *O. persica* (♦) and *O. shahpurensis* (■) in Iran.

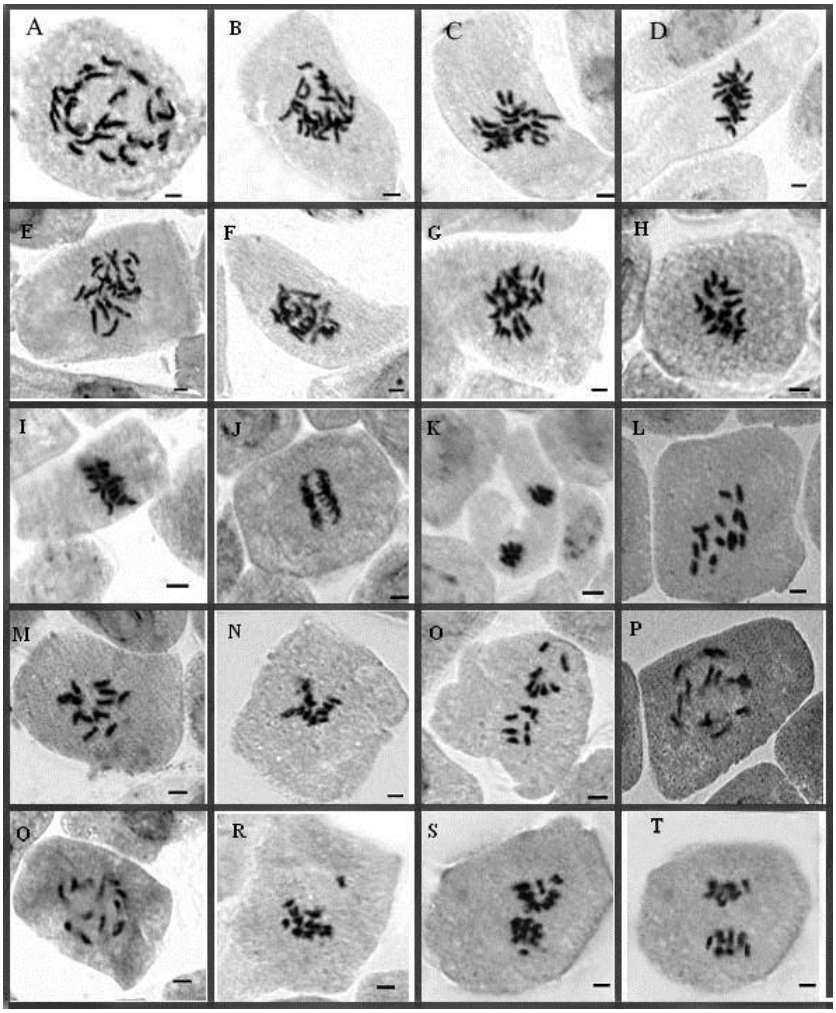

Figure 2. (A − T) Representative mitotic cells in different *Onobrychis* species: (A) Prophase in *O. viciifolia* (14214) (2*n* = 4*x* = 28), (B, C) Prometaphase in *O. viciifolia* (14214), (D) Metaphase in *O. altissima* (14209) (2*n* = 4*x* = 28), (E) Prophase in *O. altissima* (14209), (F) Prometaphase in *O. altissima* (14209), (G) Prometaphase in *O. sosnovskyi* (14208) (2*n* = 2*x* = 14), (H) Prometaphase in *O. sosnovskyi* (14212), (I) Metaphase in *O. sosnovskyi* (14212), (J) Anaphase in *O. sosnovskyi* (14208), (K) Telophase in *O. shahpurensis* (14213) (2*n* = 2*x* = 14). (L, M) Metaphase in *O. shahpurensis* (14213), (N) Anaphase in *O. shahpurensis* (14213), (O) Telophase in *O. persica* (14197) (2*n* = 2*x* = 14), (P) Prophase in *O. persica* (14197), (Q) Prometaphase in *O. persica* (14197), (R) Metaphase in *O. persica* (14197), (S, T) Anaphase in *O. persica* (14197). Scale bars = 3 µm.

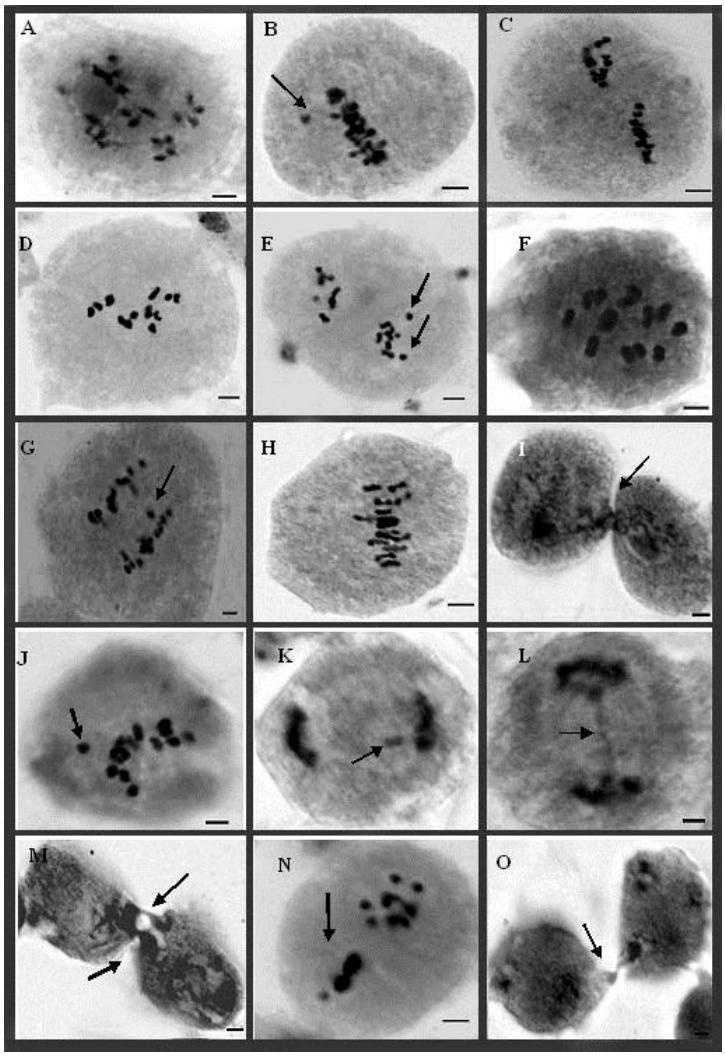

Figure 3. (A − O) Representative meiotic cells in *O. altissima* (2*n* = 4*x* = 28): (A) Pachytene (14202), (B) Metaphase I showing 14 bivalents and 1 B-chromosome (14202), (C) Metaphase II (14209), (D) Early metaphase I (14204), (E) Telophase I with laggard chromosomes (14204), (F) Early metaphase I (14192), (G) Anaphase I (14192), (H) Early metaphase I (14201), (I) Cytomixis (14537), (J) Fragmented chromosomes in metaphase I (14537), (K) Laggard chromosome in anaphase I (14537), (L) Bridge in telophase I (14537), (M) Cytomixis (14556), (N) Asynchronous nucleus in metaphase II (14556), (O) Cytomixis in telophase II (14556). Scale bars = 3 µm.

Anaphase and telophase laggard chromosomes

In this study only Kaleibar population (14204) of *O. altissima* and two populations of *O. viciifolia* showed formation of laggard chromosomes from anaphase I to telophase II (figures 3E, 3K, 7B, 7E and 7F), while other populations studied here did not form any laggard chromosomes. The highest percentage of AI/TI cells with laggard chromosomes occurred in Taham population (14198) of *O. viciifolia* (table 2).

Chromosome stickiness

Chromosome bridges resulting from stickiness were observed in anaphase I and II as well as telophase I and II stages in *O. viciifolia*, *O. altissima*, *O. shahpurensis* and *O. sosnovskyi* (figures 3L, 4F, 4G, 5F, 6B, 6D, 6F, 6I and table 2).

Desynapsis

A complete desynapsis was observed only in Taham population (14198) of *O. viciifolia* (figure 7H and table 2) and Gheidar population (14197) of *O. persica* (figure 4J and table 2).

Cytomixis

The chromatin/chromosome migration occurred in different directions from early prophase to telophase in some *Onobrychis* species and populations studied (figures 3I, 3M, 3O, 5K, 6B, 7K and table 2).

B-chromosomes

B-chromosomes or accessory chromosomes only was observed (10.64%) in Ardebil population (14202) of *O. altissima* (figure 3B and table 2).

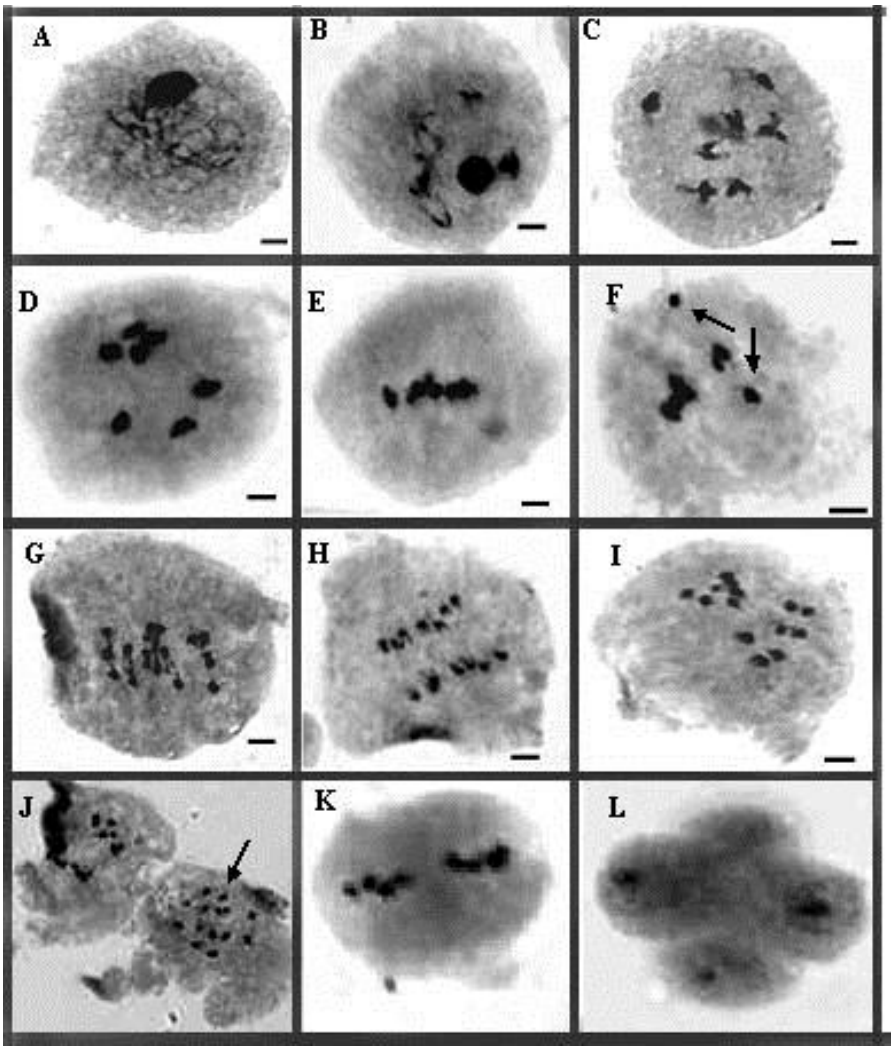

Figure 4. (A − L) Representative meiotic cells in *O. persica* (14197) (2*n* = 2*x* = 14): (A) Zygotene, (B) Pachytene, (C) Late pachytene, (D) Diakinesis showing 7 bivalents, (E) Metaphase I, (F) Metaphase I with fragmented chromosomes, (G) Early anaphase I, (H) Anaphase I, (I) Telophase I, (J) Anaphase I and desynapsis in metaphase I and 14 monovalents, (K) Metaphase II, (L) Telophase II. Scale bars = 3 µm.

Discussion

Mitotic chromosome number and ploidy level

According to Abou-el-Enain (2002), two basic chromosome numbers (*x* = 7 and *x* =8) and four ploidy levels (2*n* = 2*x* = 14, 2*n* = 4*x* = 28, 2*n* = 8*x* =56, 2*n* = 2*x* = 16 and 2*n* = 4*x* = 32) are present in the genus *Onobrychis*. Results from the present study showed that *O. viciifolia* and *O. altissima* are tetraploid with the base number of 2*n* = 4*x* = 28, while *O. shahpurensis*, *O. persica* and *O. sosnovskyi* are diploid with the base number of 2*n* = 2*x* = 14. *O. altissima* and *O. viciifolia* behave as monocarpic perennials in their natural habitats. The comparative biology of *O. viciifolia* and *O. altissima* has led to the hypothesis that these two species have a progenitor-derivative relationship with the former species having differentiated from the latter. However, *O. viciifolia* differs from it by having small teeth on the crest of pod and wings shorter than 3 mm. One ploidy level (2*n* =4*x* = 28) for *O. viciifolia*, and two ploidy levels (2*n* = 2*x* = 14 and 2*n* = 4*x* = 28) for *O. altissima* have been perviously reported (Takhtajan, 1990).

Meiotic behavior and abnormalities

The meiotic irregularities observed in the studied *Onobrychis* species showed variation and included the occurrence of varied degrees of sticky chromosomes, formation of laggards and bridge in anaphase I and II, telophase I and II, cytomixis, cytoplasmic connections, desynapsis in metaphase I and asynchronous nuclei in metaphase II. Such irregularities have been also reported previously for *O. viciifolia* and *O. altissima* of this section and also in *O. chorassanica* of *O.* sect. *Hymenobrychis* (Ranjbar et al., 2009a, 2010b, 2010c, 2010d, 2011).

Anaphase and telophase laggard chromosomes

Laggards and non-oriented chromosomes may produce micronuclei, 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), leading to the formation of micro-pollen and, probably, to gametes with unbalanced chromosome numbers (Mansuelli et al., 1995). Non-oriented bivalents may be related to impaired attachment of kinetochores to the spindle fibers (Nicklas and Ward, 1994). It has been suggested that infertility in polyploids is not solely due to the production of aneuploid gametes formed by improper segregation of chromosomes during anaphase/telophase stages, the genetic factors may also bring about pollen sterility as evidenced in different tetraploid species (Hazarika and Rees, 1967; Pagliarini, 1990, 2000; Baptista-Giacomelli et al., 2000).

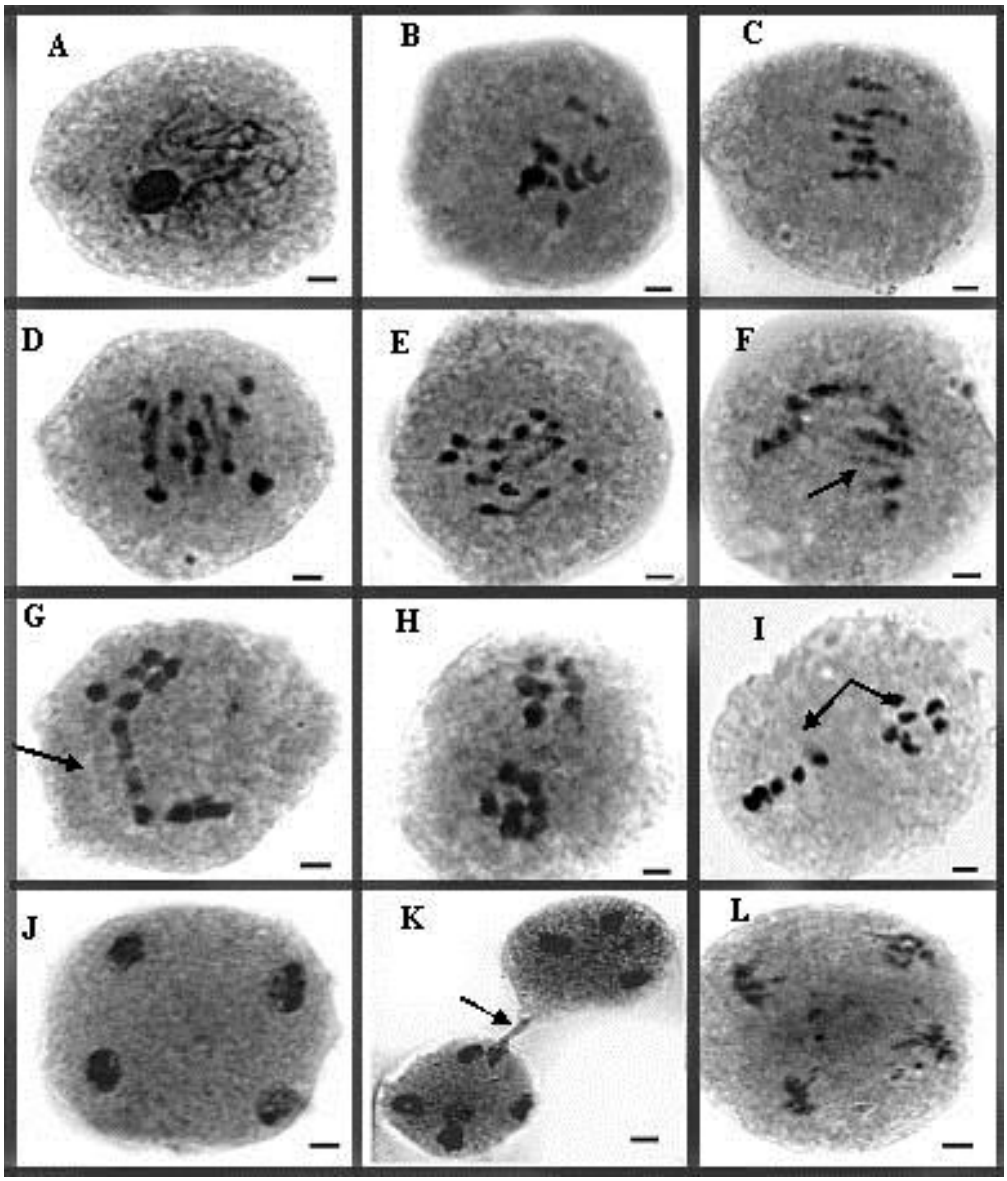

Figure 5. (A − K) Representative meiotic cells in *O. shahpurensis* (19182) (2*n* = 2*x* = 14): (A) Zygotene, (B) Diakinesis showing 7 bivalents, (C) Metaphase I, (D) Early anaphase I, (E) Anaphase I, (F) Bridge in anaphase I, (G) Bridge in late anaphase I, (H) Telophase I, (I) Anaphase II, (J) Telophase II, (K) Cytomixis in telophase II and pentapolar cells. Scale bars = 3 µm.

Table 1. Taxa and acronyms examined in this study.

Species	Voucher No.	Locality	Alt. (m)	Collector name
O. viciifolia	BASU 14214	West Azerbaijan: Orumieh to Oshnavieh, after Sangar	1650	Ranjbar & Hadadi
O. viciifolia	BASU 14198	Zanjan: Taham	2000	Ranjbar & Hadadi
O. viciifolia	BASU 14529	East Azerbaijan: Hashtrood to Maraghe, 70 km to Maraghe	1529	Ranjbar & Hadadi
O. viciifolia	BASU 14544	Tehran: Polur to Firuzkuh, 5 km after railway	1571	Ranjbar & Hadadi
O. viciifolia	BASU 14527	East Azerbaijan: Hashtrood to Maragheh, 80 km to Maragheh	1617	Ranjbar & Hadadi
O. shahpurensis	BASU 14213	West Azerbaijan: Salmas to Orumieh, Ghooshchi neck	1870	Ranjbar & Hadadi
O. shahpurensis	BASU 19182	West Azerbaijan: Salmas to Kozerash, Kozerash	2225	Ranjbar & Hadadi
O. sosnovskyi	BASU 14208	East Azerbaijan: Kharvanak	1215	Ranjbar & Hadadi
O. sosnovskyi	BASU 14212	East Azerbaijan: 16 km to Marand	1725	Ranjbar & Hadadi
O. altissima	BASU 14209	East Azerbaijan: 10 km after Varzaghan, Mirzaali Kandi	2010	Ranjbar & Hadadi
O. altissima	BASU 14202	Ardebil: Ardebil	1540	Ranjbar & Hadadi
O. altissima	BASU 14201	Ardebil: Saein neck	1770	Ranjbar & Hadadi
O. altissima	BASU 14204	East Azerbaijan: Kaleibar	1650	Ranjbar & Hadadi
O. altissima	BASU 14537	East Azerbaijan: Khoy, Ghotor road, 2 km to Darekhan	1357	Ranjbar & Hadadi
O. altissima	BASU 14556	Khorasan: Bojnourd to Esfarayen, Assadly neck	1800	Ranjbar & Hadadi
O. persica	BASU 14197	Zanjan: Gheidar	1950	Ranjbar & Hadadi

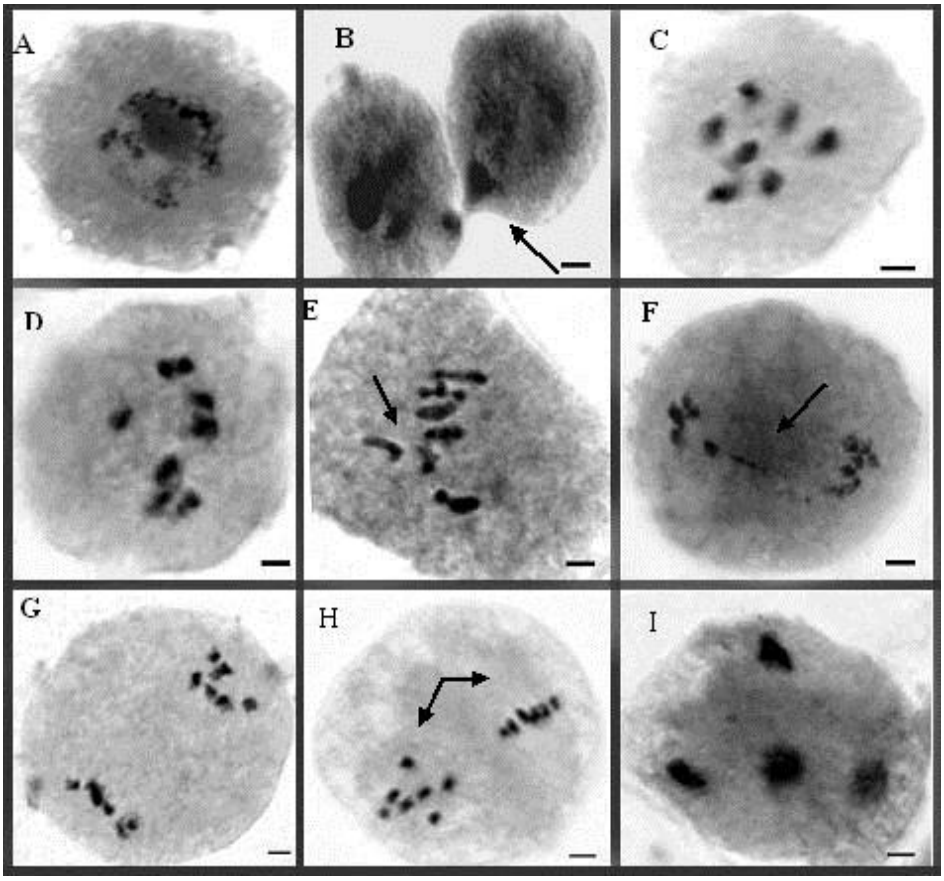

Figure 6. (A − I) Representative meiotic cells in *O. sosnovskyi* (2*n* = 24*x* = 14): (A) Zygotene (14208), (B) Cytomixis in zygotene (14208), (C) Diakinesis (14208), (D) Diakinesis showing 7 bivalents and (14212), (E) Metaphase I with fragmented chromosome (14212), (F) Bridge in anaphase I (14212), (G) Telophase I (14212), (H) Asynchronous nucleus in metaphase II, (I) Telophase II. Scale bars = 3 µm.

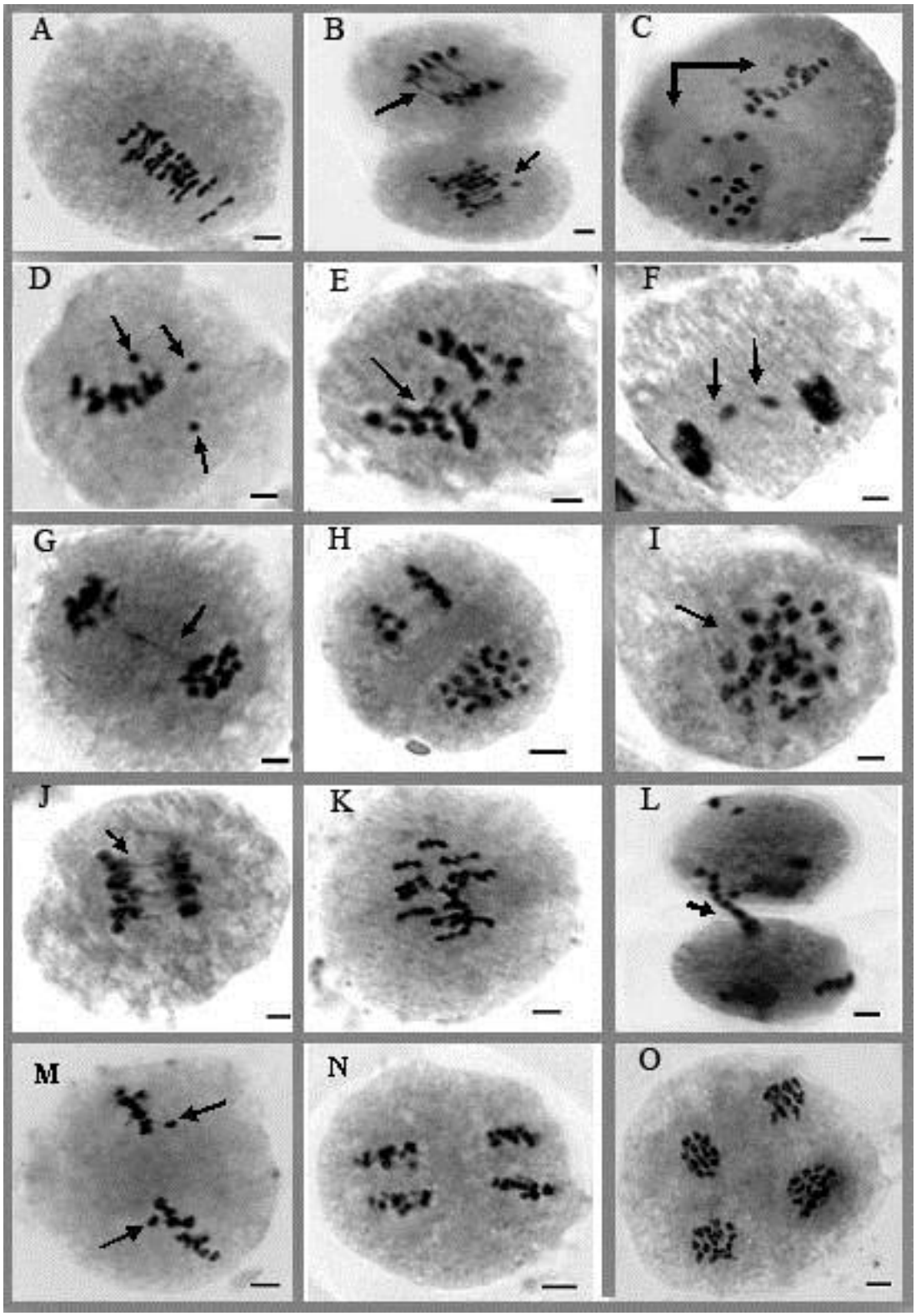

Figure 7. (A − O) Representative meiotic cells in *O. viciifolia* (2*n* = 4*x* = 28): (A) Early metaphase I (14198), (B) Bridge and laggard anaphase I (14198), (C) Asynchronous nucleus in metaphase I (14198), (D) Fragmented chromosomes metaphase I (14529), (E) Laggard chromosomes in anaphase I (14529), (F) Laggard chromosomes in telophase I (14529), (G) Bridge in telophase I (14529), (H) Asynchronous nucleus in metaphase II (14544), (I) Desynapsis (14544), (J) Bridge in anaphase I (14544), (K) Metaphase I (14527), (L) Cytomixis and laggard chromosomes in metaphase II (14527), (M) Fragmented chromosome in metaphase I (14527), (N) Anaphase II, (O) Telophase II (14527). Scale bars = 3 µm.

Table 2. Meiotic beheavior in different populations of *O viciifolia, O. altissima*, *O. shahpurensis*, *O. sosnovskyi*, and *O. persica.*

Taxa	sos 14208	sos 14212	shp 14182	alt 14202	alt 14201	alt 14204	alt 14209	Alt 14537	alt 14556	alt 14527	vic 14554	vic 14529	vic 14214	vic 14199	vic 14198	per 14197
% Z/P	42.3	26.24	52.7	9.88	17.25	33.14	54.72	66.95	30.37	52.15	70.83	3.27	31.85	9.07	66.9	54.49
% Cytomixis/Cytoplasmic connection	3.33	0	0	0	3.1	0	1.19	5.01	4.87	5.17	2.45	4.52	13.78	0	5.25	2.96
% D/M I	30.12	20.28	18.9	25.97	28.18	13.31	1.31	19.52	4.9	10.45	18.4	14.73	18.12	4.47	12.91	21.03
% Cytomixis/Cytoplasmic connection	3.62	0	0	6.84	12.96	0.65	0	7.48	9.46	7.09	8.44	11.76	4.52	0	4.77	3.77
% Fragmented / Forward chromosome	2.12	0	0.81	0	0	0	0	2.26	2.36	0	0.67	9.47	2.5	0	0	3.94
	0	0	0	10.64	0	0	0	0	0	0	0	0	0	0	0	0
0%0 A I/T I	3.16	9.16	6.95	9.2	22.07	2.91	8.3	8.41	10.51	3.71	3.91	3.61	11.19	7.03	8.4	3.95
% Cytomixis/Cytoplasmic connection	0	0	0	0	7.31	0	0	10.52	17.67	0.09	0	13.33	20.6	12.7	6.21	1.6
% Laggard chromosome	2.12	0	43	0	0	7.69	0	3.67	2.67	5.45	15.82	10.11	0.26	5.45	17.51	2.12
% DS	1.1	0	0	0	0	0	0	0	0	0	1.12	0	0	0	0	1.06
% P II	5.45	0	7.89	17.88	1.28	6.47	10.88	11.5	9.32	10.79	0.62	6.98	10.56	14.7	1.8	4.59
% Cytomixis/Cytoplasmic connection	26.2	0	0.83	5.43	8.33	0	1.6	15.76	20.56	11.8	0	17.93	19.68	8.69	21.05	29.3
% M II	1.58	2.22	0.66	2.61	4.39	2.69	5.28	3.8	13.55	5.53	0.06	0.62	5.01	3.7	0.47	1.69
% Cytomixis	0	0	0	7.31	0	0	0	3.48	7.92	3.65	0	15.38	16.8	7.6	0	2.04
% Laggard chromosome	0	0	0	0	0	0.2	0	0	1.97	7.31	0	0	16	0	0	0
% Asynchronous nucleus	35.7	4	0	26.8	17.14	0	9.09	2.32	10.06	9.75	0	0	0	0	0.6	14.28
% A II/T II	17.84	49.97	13.5	34.43	26.79	26.79	19.49	8.277	26.1	17.34	6.21	9.58	24.13	61.38	9.49	28.83
% Cytomixis/Cytoplasmic connection	6.32	2.07	0	1.6	10.8	10.8	3.18	12.29	8.56	12.48	0	1.25	18.19	2.08	0	5.7
% Laggard chromosome	0.84	0.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
Ploidy	2	2	2	4	4	4	4	4	4	4	4	4	4	4	4	2

Abbreviations: Z/P = Zygotene/Pachytene; D/MI = Diakinesis/Metaphase I; *n* = Chromosome number; AI/TI = Anaphase I/Telophase I; PII = Prophase II; MII = Metaphase II; AII/TII = Anaphase II/Telophase II.

Chromosome stickiness

Sticky chromosomes were observed from early stages of prophase till the final stages of meiosis in some populations studied. The thickness of bridges observed and the number of chromosomes involved in their formation varied among different meiocytes and the species studied. Chromosome stickiness may be caused by genetic and environmental factors, and several agents have been reported to cause chromosome stickiness (Pagliarini, 2000).

Desynapsis

Desynapsis occurs either due to the action of recessive ds genes in a homozygous situation or early chiasma terminalisation which may lead to the formation of meiocytes with double normal chromosome number. In several cases such univalents may have difficulty during anaphase I movement and become lagged therefore producing aneuploid gametes causing reduction ion pollen fertility of plants. However, they may skip the first anaphase and form restitution nucleus resulting in the formation of unreduced gametes as reported in some other species (Veilleux, 1985; Sheidai et al., 2006, 2007).

Cytomixis

The chromatin/chromosome migration occurred in different directions from early prophase to telophase in the *Onobrychis* species and populations studied (figures 3I, 3M, 3O, 5K, 6B, 7K and table 2).

B-chromosomes

B-chromosomes that occur in addition to the standard or A-chromosomes in some of the plants, are smaller than other chromosomes and do not form any association with them, although they could arrange themselves along with the A-chromosomes on the equatorial plane of the spindle and move to the poles during anaphase. In some cases they occurred as laggard chromosomes. The significance of B-chromosomes is to be found in their widespread occurrence in hundreds of flowering plants, and also in gymnosperms and in some lower forms such as ferns, bryophytes and fungi (Jones and Rees, 1982).

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Title. In the first page, papers should be headed by a concise and informative title. The title should be followed by the authors' full first names, middle initials and last names and by names and addresses of laboratories where the work was carried out. Identify the affiliations of all authors and their institutions, departments or organization by use of Arabic numbers (1, 2, 3, etc.).

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Table of Contents

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