Karyotype analysis of the micropropagated black berry (*Rubus fruiticosus*)

Mohamed Mohamed Abd Alla[1], and Aisha Mohamed Abd Elkawy[2]

[1]Assistant prof. Of Biotechnology. Desert Research Center Cairo, Egypt
Email: alaarabimemo@yahoo.com
[2]Jasan Uni. Faculty of Science,biology department, KSA. First address: Faculty of Science, Elazhar Uni. Cairo, Egypt

Abstract

Black berry *(Rubus fruiticosus)* plantlets were in vitro propagated. Samples from root tips of the rooted plantlets and the mother plant were excised for cytogenetic analysis. The karyotype analysis revealed that there were 14 autosomal chromosomes (7 bivalents) in C-metaphase profile. The chromosomes were short and their length was in average between 5.88µ to 3.027µ and the chromosome area was in range between 6.83µ² to 3.547 µ². The chromosomes were metacentric chromosomes and the positions of their centromeres were found at the points from 48.868% to 47.473%. This study proved that no significant variations were observed between the tissue culture derived plantlets and their mother plant in the chromosome number, length, and neither area nor centromere position by the karyotype analysis.

Keywords: rubus fruiticosus, tissue culture, karyotype

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Introduction

Rubus fruiticosus belonged to the family Rosacea, grows well with low shilling requirements, it has a good quality and high yield of fruit production. It was considered as a medicinal plant. It contains an anti-oxidant and anti-cancers pigments which are found in the fruits (anthocyanin and hexothancenes). Tissue culture technique is an excellent method to produce huge amounts of micropropagated plantlets in a few time and limited place. This study was performed to determine the genetic stability by studying the chromosomal changes that can be occurred through the micropropagation process. Somaclonal variations were the most important problem which can be avoided by using the biotechnological diagnosis (molecular

markers, biochemical markers and Karyotype analysis).Orton (1980) found that, spontaneous polyploidy, aneuploidy, and chromosomal rearrangements were observed in callus and suspension cultures of *Hordeum vulgare, H. jubatum,* and their interspecific hybrid. The extent to which each class of chromosomal variability was present in a culture depended upon differentiated state, age, and history. Cytological and isozymic analysis of subdivided callus cultures revealed spatial segregation of chromosomal variability. Cytogenetic analyses were performed to determine the expression of this in vitro chromosomal variability in corresponding regenerated plant tissues. A cytological study has been made of plants regenerated from cultured immature embryos of four wheat cultivars (*Triticum aestivum*) by Karp and Maddock (1984), they found that, in about 29% of the plants examined were aneuploidy with a range in chromosome numbers of 38–45. Sandroval *et al*,(1996) during their study on the micropropagation of banana (*Grand naine*), they found that some cells of the micropropagated plants were found to have an abnormal chromosomes number and there were an aneuploidy percentage from 5%:35% in the micropropagated plants. Rani and Raina (2000) reported that the concept of genetic uniformity among micropropagated plants derived through organized meristems was exploded by several convincing reports of the incidence of somaclonal variation at morphological, cytological (chromosome number and structure), cytochemical (genome size). Somaclonal variation is not limited to any particular group of plants. Wang *et al* (2000) reported that mixploid variation had a great effect on the variation in the production of banana vitro plants, the rate of the chromosomal aberration in cells was characterized by starting at a much higher level and rising more rapidly in the preceding 15 subcultures as compared with the control and the mixploid variant can cause the rate of variation to rise during micropropagation. Karyotype is a test to identify and evaluate the size, shape, and number of chromosomes in a sample of plant cells. Abnormal positions of chromosome pieces can cause problems with plants growth, development, and fruits production. Karyotyping is done to determine whether the chromosomes of plant have an abnormality that can be occurred during micropropagation, determine whether a chromosome defect is present. Sumita and Salysh (2003) studied the changes in chromosome number through micropropagation of Holarrbena antidysenterica as well as changes in DNA content using cytophotometric data, they found that somatic chromosomes from the micropropagated plants and the in vivo plants revealed a diploid chromosomes and no changes in ploidy level were observed which confirm priviouly reported finding that cultured regenerated plants were clonaly uniform and laked somaclonal variation. Tygi and Kancherla (2003) they found in their study on chromosome number of *Panadorea pandorana* micropropagated plants and the nature plant that, no differences in chromosome number although the tissue culture plants looked different morphologically as compared with naturally grown plants. Chengqi (2008), determined somatic chromosome numbers individuals of *Allium przewalskianum* from the Qinghai-Tibet Plateau and five populations were selected for karyotype analysis based on the available chromosome data. Fernandes *et al*. (2009) studied the karyotypes of four South American species of *Cestrum* using conventional staining. Kaur *et al* (2009) when they studied the genetic stability of the micropropagated plants of *Gentiana kurro*

using Karyotype analysis that found there were no chromosomal variations in the micropropagated plants through nodal segments culturing. The same results were obtained by AbdAlla and Aisha (2010) on date palm *(Phoenix dactylifera*) when they studied the chromosome number, chromosome size and centromer position of the micropropagated date palm plantlets and the mother plant, they found that no chromosomal aberrations and no significant differences were found between plantlets and their mother plant. Chatterjee and Ghosh (2012, *and a*) observed that the callus growing in medium containing NAA revealed different degree of ploidy and mitotic abnormalities such as stickiness, clumping, diplochromatids and spindle disturbances from the early stage and the mitotic index was also recorded. The frequency of chromosomal abnoramlities gradually increases with the age of the callus tissues increases. They also found that IAA has a distinct role to play in influencing Karyological instabilities and mitotic rate of cells and also observed the role of different hormones in inducing karyological changes during in vitro growth. Pradhan and Das (2012) used chromosome analysis to study the genetic variation of different ecotypes of mangrove associate *Suaeda nudiflora* that obtained from Bhitar kanika mangrove forest of Orissa. Zhou *et al* (2012) examined the genetic diversity of four new species related to south western Sichuan buckw heats using karyotypes. Beena, V.L. and Karyotypes and SDS-PAGE analysis in three accessions were used to access the genetic diversity by using root tip cells of *Passiflora edulis* Beena and Beevy, (2015).

This study was carried out aiming to determine the chromosomal aberrations that can occurred during micropropagation process for blackberry *Rubus fruiticosus* tissue culture derived plantlets.

Materials and methods

Plant material

This study was carried out in Tissue Culture Laboratory, North Sinai Research Station, Genetic Resources Department, Desert Research Center, Cairo, Egypt; through the years 2011-2012. The micropropagated *Rubus fruiticosus* plantlets were produced using stem segment technique through the micropropagation study for this variety. The micropropagated plantlets were acclimatized in the green house at North Sinai Research station. Root samples from the *in vitro* plantlets were collected to assess the cytological changes between the mother plant and the micropropagated plantlets through micropropagation process. Cytological studies and karyotype are based on the morphological characteristics of chromosomes visualization. Karyotype analysis is a well established method (Fukui and Kakeda, 1994). The excised root tips were chemically treated before microscope utilization as follow:

- Collection of lateral root tip from both (tissue culture rooted plantlets and mother plant
- - Pretreatment with colchicines (0.25%) for 2h at room temperature.
- Root tips were fixed in fixation solution (Ethanol alcohol and Glacial acetic acid 3:1) at 4°C for 5 min. then were washed with distilled water.
- The root tips (2-3mm) were then incubated in enzyme mixture of (4% cellulose and 1% pectenase, 75 mM KCl and 7.5 mM EDTA) on the glass slides at 37°C for 40 min.
- The root tips were washed with distilled water to remove enzymatic mixture and then root tips were squashed with a drop of Aceto Orcein stain after they were flamed.
- The prepared samples were then examined on microscope using Image Processing Analysis System (Mac-Type).

The cytological profiles of the divided root tips cells of the tissue cultured plantlets and the mother plant samples were imaged by digital camera in the c-metaphase and analyzed using Image Processing analysis system (Video Test Karyotype). The characteristics data for each chromosome of the three replicates (R1. R2 and R3) of *Rubus fruiticosus* root samples were: (chromosomes number, chromosome lengths (µ), Chromosomes area (µ²) and centromeric index percentage(length of short arm/ chromosome length). The chromosomes were arranged according their lengths in a Karyotype according to Hussein (2005). For Karyotype analysis of *Rubus fruiticosus* the two homologues chromosomes (a and b) of each chromosome pair were judged according to length similarity of short arm, long arm, the total length and centromeric index percentage. An average length, area and centromeric index were calculated (a+b)/2 for each chromosome pair was determined and arranged in descending order and were given their number from 1 to 7.

Means values were calculated using the obtained data from each of the three replicates of the tissue cultured plantlets and the mother plants. Mean values of the samples were evaluated by using T test for paired observation, according to formula: $tc = (d` - M_0) / sd`$.

tc = t calculated value, d`= mean of differences

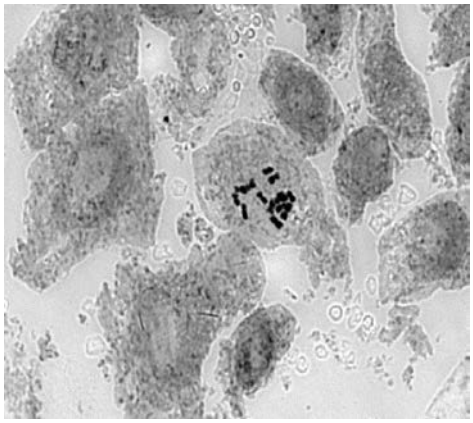
Results and Discussions

The results of the cytological analysis using the root tips of the micropropagated blackberry (*Rubus fruiticosus*) plantlets and their mother plant revealed that, the root tip cells were containing 14 chromosomes which were paired in 7 bivalents in C-metaphase profile as clear in table (1) and Fig.(1). Data in table (1) shows the chromosomes lengths by which they were arranged asthey were clear in figure (2). The highest chromosome length was 5.88µm followed by 5.54µm for chromosomes No 1 and No 2 respectively. The length of chromosome No.3 was 4.851 µm and chromosome No 4 was 4.343 µm, while the length of chromosome No.5 was

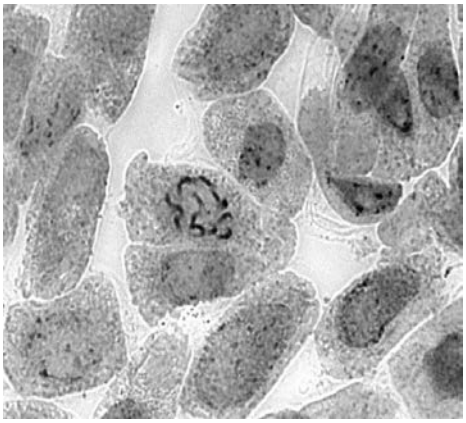
3.617 µm and chromosome No 6 was 3.54 µm, while the length of chromosome No.7 was the smallest (3.03µm). There were no significant differences in chromosome number neither in the micropropagated plants nor their mother plant. They contained 7 chromosomes in their cells and also the comparison between the micropropagated plantlets and the mother plant showed no significant differences in the chromosomes lengths. The measurements of chromosomes area (µ²) in table (1) indicate that *Rubbus fruiticosus* chromosomes are small and the difference between the big chromosome and the smallest chromosome is not large. The area (µ²) of the big chromosome was 6.83µ² followed by 6.51 µ²and 5.62µ²for chromosome No 1,2 and 3 respectively.

Table (1): The characterization of *Rubus fruiticosus* chromosome of the micropropagated plants and their mother plant

Chromosome No	Chromosome length µm		T calculated	Chromosome Area µ²		calculated T	Centromer position		calculated T.
	Micr.	Mother		Micro.	Mother		Micro	Mother	
1	**5.88**	**5.88**	**1.871**	**6.83**	**6.83**	**0.00001**	**47.94**	**47.936**	**0.31**
2	**5.54**	**5.54**	**1.871**	**6.51**	**6.51**	**0.00001**	**48.87**	**48.868**	**0.023**
3	**4.853**	**4.851**	**1.866**	**5.62**	**5.617**	**0.215**	**48.29**	**48.29**	**0.000013**
4	**4.343**	**4.343**	**1.871**	**4.85**	**4.847**	**0.215**	**48.27**	**48.268**	**0.0233**
5	**3.617**	**3.613**	**1.643**	**4.47**	**4.467**	**0.215**	**47.476**	**47.473**	**0.143**
6	**3.54**	**3.537**	**1.870**	**3.61**	**3.607**	**0.215**	**48.127**	**48.125**	**0.0233**
7	**3.03**	**3.027**	**1.871**	**3.55**	**3.547**	**0.215**	**48.183**	**48.181**	**0.0233**



Micropropagated plantlets



Mother plant

Figure (1) the cytological profile of *Rubus fruiticus* tissue culture derived plantlets and their mother plant in metaphase position.

The other four chromosomes areas were 4.85µ²,4.47µ²,3.61µ²and 3.55µ² respectively as they were arranged in table (1)and Fig(3) for chromosomes No 4, No 5, No 6 and No 7. Data in table (1) illustrating the centromer position of *Rubbus fruiticosus* chromosomes (the ratio of the short arm/ the chromosome length). The centromer position of chromosome No.1 was at the point 47.94%,and also chromosome No.5 was at the point 47.47% of the chromosome length. While the other five chromosomes areas were 48.87%, 48.29%, 48.27%, 48.127 and 48.123 % of length of the chromosomes No. 2,3,4,6, and 7 respectively. The karyotype analysis data of the micropropagated plants and the mother plant of *Rubbus fruiticosus* revealed that these chromosomes are metacentric chromosomes when the centromer position was at the point from48.847 %to 47.506% of the chromosome length and also the chromosomes were short and their lengths were ranging from 5.88 µm to 3.01µm.

These results are in agree with Wang *et al* (2000) they reported that, the rate of the chromosomal aberrations in cells were characterized by starting at a much higher level and rising more rapidly in the preceding 15 subcultures as compared with the control. Karyotype is a test to identify and evaluate the size, shape, and number of chromosomes in a sample of plant cells. The abnormal positions of chromosome pieces can cause problems with plants growth, development, and fruits production. Karyotyping is done to determine whether the chromosomes of plant have an abnormality that can be occurred during micropropagation, determine whether a chromosome defect is present. Sumita and Salysh (2003) studied the changes in chromosome number through micropropagation of Holarrbena antidysenterica as well as changes in DNA content using cytophotometric data, they found that somatic chromosomes from the micropropagated plants and the in vivo plants revealed a diploid chromosomes and no changes in ploidy level were observed which confirm priviously reported finding that cultured regenerated plants were clonaly uniform and lacked somaclonal variation. Tygi and Kancherla (2003) they found in the study on chromosome number of *Panadorea pandorana* micropropagated plants and the nature plant that, no differences in chromosome number although the tissue culture plants looked different morphologically as compared with naturally grown plants. While Kaur *et al* (2009) found that the Karyotype analysis showed no chromosomal variations in the micropropagated plants through nodal segments culturing when they studied the genetic stability of micropropagated plants of *Gentiana kurro* using Karyotype analysis. The same results were obtained by AbdAlla and Aisha (2010) on date palm *(Phoenix dactylifera*) when they studied the chromosome number, chromosome size and centromeric position of the micropropagated plantlets and the mother plant, they found that no chromosomal aberrations and no significant differences were found between plantlets and their mother plant. Pradhan and Das(2012) used chromosome analysis to study the genetic variation of different ecotypes of mangrove associate *Suaeda nudiflora* that obtained from Bhitarkanika mangrove forest of Orissa.

These results didn't agree with Karp and Maddock (1984) they found in a cytological study had been made on plants regenerated from cultured immature embryos of four wheat cultivars (*Triticum aestivum*, 2n = 6x = 42), that ,about 29% of the plants examined were aneuploidy with

a range in chromosome numbers of 38–45 and also Sandroval *et al*,(1996) they found on their study on the micropropagation of banana (*Grand naine*) that, some cells of the micropropagated plants were found to have an abnormal chromosomes number and there were an aneuploidy percentage in the micropropagated plants. Through the micropropagation process, no changes were recorded or any degree of ploidy and mitotic abnormalities such as stickiness, clumping, diplo-chromatids and spindle disturbances from the early stage of mitotic division, so that these results disagree with that obtained with Chatterjee and Ghosh (2012, *and a*) whose observed that the callus growing in medium containing NAA or IAA revealed different degree of ploidy and mitotic abnormalities such as stickiness, clumping, diplo-chromatids and spindle disturbances from the early stage and the mitotic index was also recorded. The frequency of chromosomal abnormalities gradually increases with the age of the callus tissues increases. Beena and Beevy (2015) found that karyotype analysis suggests variations at the intra-as well as interspecific levels in Passiflora.

The conclusion is that tissue culture technique helps in the micropropagation of the valuable and important by producing huge amounts of plantlets in a short time and limit are, but if many subcultures was applied it can cause some changes in the molecular, biochemical levels, in chromosomal level (degree of ploidy and mitotic abnormalities such as stickiness, clumping, diplo-chromatids and spindle disturbances from the early stage of mitotic division) and also somaclonal variations can be occurred. So that, to avoid the Somaclonal variations, micrpopropagation process should stop after a few successive subcultures and begin with a new explants.

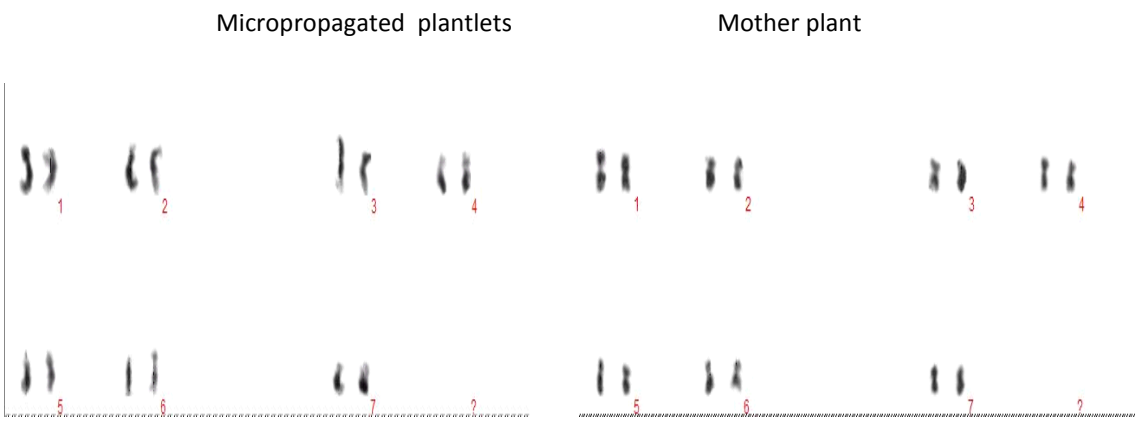


Figure (2): The chromosome number, length of the micropropagated *Rubus fruiticosis* and their mother plant.

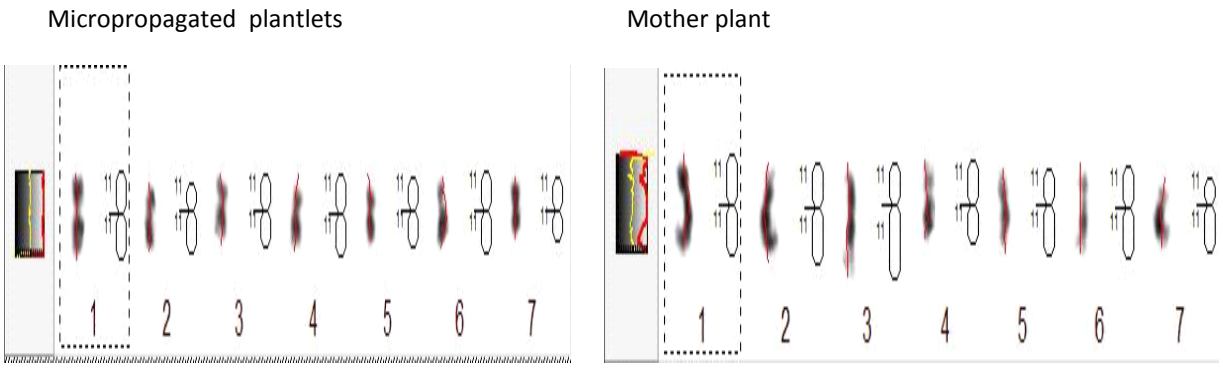


Figure (3): The chromosome area and centromeric position of the micropropagated *Rubus fruiticosis* and their mother plant chromosomes.

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