Effect of the Two Biofertilizers Rhizobacterien and Phosphoren on Cell Cycle and Mitotic Division in *Vicia faba*

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Summary The present investigation has been conducted to study the mutagenic effect of the 2 biofertilizers Rhizobacterien and Phosphoren on mitotic division in root tips of *Vicia faba*, addition to the cell image was used to estimate and analyze the gray values of these cells.

The 2 applied biofertilizers induced a significant and/or highly significant increase in mitotic activity. Proliferation activity was activated with the increase of the biofertilizers treatments. Moreover, the percentage of cells having 2C-DNA content (G_0/G_1) decreased than those of control, while the percentage of the cells 3C (S phase) and 4C (G_2 , M) increased than control at cell image analysis. On the other hand, the biofertilizers induced several chromosomal aberrations in mitotic cells.

Key words Biofertilizers, Proliferation activity, Cell image analysis.

Plant production in many countries has increased due to the use of high yielding cultivars and enhanced consumption of inorganic fertilizers represents the nitrogenous one. The excessive use of nitrogen fertilizers represents the major cost in plant production and creates pollution of agroecosystem as well as deterioration of soil fertility, Fisher and Richter (1984).

As a result of dangerous effects of chemical fertilizers, many scientists directed their attention to follow other safety and sustainable agriculture that is the gradual replacement of chemical fertilizers by biofertilizers. So we directed out attention to follow the mutational and cytogenetic effects of some biofertilizers (Rhizobacterien, Phosphoren) on root tip cells of *Vicia faba* plants with microscopically and cytometrically analysis in mitotic division.

During the last few years image cytometry has been progresavily used as a more convenient, more rapid and more efficient method to measure nuclear DNA in cell nuclei, Hammatt *et al.* (1991).

Kiss *et al.* (1992) reported that, one application of image cytometry is quantitating DNA amount and its intercellular and intercellular distribution in normal and abnormal cells. Moreover, it is possible with this method to perform cell cycle analysis and study its regulation in plant cell cultures (Zhang *et al.* 1992, Glab *et al.* 1994).

Moustafa (1992) recorded that the main kinetic parameters of cell proliferation study is the length of the cell cycle and the duration of its phases, as well as, S fraction (percentage of cell in S phase) and growth fraction (sum of cyclic cells).

Shehab *et al.* (2000) studied the effect of Microbein biofertilizer on cytomorphological alteration and image analysis of cell proliferation of *Vicia faba*. They found that microbein biofertilizer induced a significant increase in mitotic activity that is associated with an increase in the amount of DNA content and decrease in the amount of RNA and protein contents. Proliferation activity was enhanced with the increase of microbein treatments. Moreover, the biofertilizer induced several chromosomal aberrations in mitotic cells.

Materials and methods

These studies were carried out on broad bean, seeds of pure strains of *Vicia faba* L. (C.V. Giza3), obtained from the Crop Research Institute, Agriculture Research Center (ARC), Giza, Egypt. It has economical importance and it is one of the most commonly used plant material for Cytological studies.

Also 2 biofertilizers were used, which adopt active policies toward development of Egyptian agriculture.

Rhizobacterien: A mixture of nitrogen fixing bacteria (*Rhizobium* sp., *Azospirillum* sp., *Azotobacter* sp., Abu-Zekry 2000) used for different crops. The recommended dose is 200 g/fedan.

Phosphoren: It contains raw phosphate dissolving bacteria, where the recommended dose is 200 g/fedan.

Two types of experiments were carried out to study the effect of the biofertilizers.

1) Seeds of equal size were mixed with different doses of the biofertilizers (1/2R, R, 2R) then germinated on petri dishes containing cotton wool saturated with water, where R equal to 1.6 g/100 seeds.

2) Another group of seeds were mixed with the lowest dose 1/2R for different times (1, 2, 10 h), then germinated as above. As the root length was about 1.5–2 cm the roots were cut, fixed and stored in 70% alcohol for cytological studies. The control was simultaneously treated with water. Five roots from each treatment and control were stained by the Feulgen technique (Darlington and Lacour 1976). Mitotic index (MI), total percentage and type of mitotic abnormalities were calculated.

Statistical analysis of cytological data was conducted using t-test.

Nuclear image analysis and cell cycle classification: Cytometric determinations generate histograms for cell distribution according to DNA content using treated root tip cells with different biofertilizer concentrations compared with the control. Permanent preparations of Feulgen-stained root meristems were used in this assay.

Image analysis of the root cells nuclei was performed using Leica quantiment 520 apparatus. The organization, hardware and software package of any analyzer have been described by Brugal (1984).

The cell populations were characterized by the histograms of cell distribution according to their nuclear DNA content as expressed by integrated optical density (IOD). Based on the estimation of DNA of the nucleus, DNA contents, the fraction of cells histogram in 2C, 3C, and 4C can be estimated using Dean method (1980). Undergoing different phases of cell cycle were calculated as a percentage using Moustafa *et al.* (1988). These include, cells with DNA amount less than 2C value, cells with 2C DNA ($G_0 + G_1$), cells with 3C DNA (S phase), cells with 4C DNA (G_2 + polyploidy phases), cells with DNA amount more than the 4C value (polyploidy, proliferation fraction ($S + G_2 + M$)). The differentiation of chromatin activity as a response of Feulgen reaction can be demonstrated on monitor color using (Garbay *et al.* 1981).

Results and discussions

Cytophotometric analysis

The effect of Rhizobacterian and Phosphoren biofertilizers was clear as Figs. 1, 2, where there is 2 peaks. The first peak corresponds to 2C mean diploid distribution of DNA content which is nearly at 400 A.U., *i.e.* equal to (24.5 pg) (Van't Hof 1974). All values of treated and untreated root tip cells around 400 A.U., represent cells in anaphase 2C, telophase, G_1 -post mitotic and G_0/G_1 .

The second peak in the same Figs. 1, 2 was the DNA class and corresponds to G_2 , prophase, metaphase and anaphase 4C proliferating cells. The intermediate values present between 2C and 4C

Fig. 1. DNA histograms of Rhizobacterien biofertilizer with different doses, time of treatment and control.

I.O.D.: Integrated Optical Density

A.U.: Area of unit (arbitrary units)

Fig. 2. DNA histograms of Phosphoren biofertilizer with different doses, time of treatments and control.

I.O.D.: Integrated Optical Density

A.U.: Area of unit (arbitrary units)

DNA with a content ranging from approximately 450–750 A.U. correspond to cells in S phase.

Few number of meristematic cells of treated *Vicia faba* contained excess nuclear DNA content over 4C at A.U. ranging from 950–1300 A.U. In contrast, there were some cells with DNA content less than 2C value. So the biofertilizers caused a marked variation in the proportion of the cells according to the DNA content (diploid, tetraploid, intermediate groups) compared with control.

Moustafa *et al.* (1988) reported that each DNA histogram can be classified into G_0 , G_1 , S, G_2 and mitotic phases adding to proliferation activity at the treated and untreated cells.

Tables 1, 2 show that, the percentage of cells at G_0 and G_1 phases have a negative relationship with increasing dose and time of treatments, and the maximum values were 41.28 and 24.5% at the low dose 1/2R of Rhizobacterien, also 47.26 and 28.16% at 1/2R and R doses of Phosphoren compared with 49.65 and 25.95% of control.

Moreover, the percentage of S phase increased with increase of Rhizobacterien and Phosphoren doses and time of treatments. While the percentage of cells at G_2 deviate slightly from control. The percentage of cells at the mitotic phases and the proliferation activity were higher than the control.

The previous results cleared that, about 51–62% from cells for Rhizobacterien followed by 50–59% for Phosphoren pass through the cell cycle at all treatments compared with control 49.4%. So, Rhizobacterien was more biologically active than Phosphoren then control.

These results agree with that observed in Figs. 3–5 from treated cells with R dose of Rhizobacterien and 1/2R dose of Phosphoren compared with control respectively, where the chromatin displays a various degrees of condensation, and that indicate the DNA activation leading to replication. This degree of condensation appeared as different colors (red color=euchromatin, yellow color=heterochromatin). The intensity of the color may depend on the amount of DNA. The relative amount of euchromatin was very high *i.e.* the red color takes a big space compared with both all chromatin at the same picture. Moreover, the relative amount of heterochromatin was lower than the control through them and all chromatin at the same pictures.

The events that drive the cell into mitosis are as follows: Mitotic cyclin accumulates gradually during G_2 , and binds to CDK to form a complex known as M-phase promoting factor (MPF). This complex is at first inactive, but through the action of other enzymes that phosphorelate and dephos-

Table 1. Mitotic phases and changes in the cell cycle phases (G_0 , G_1 , S, G_2) in both treated with Rhizobacterien and untreated groups

Doses and gray of cells at different factores	Control	1/2R	R	2R	10 min	1 h	2 h
Nicrotic cells	0.000	0.000	0.000	0.000	0.958	0.125	2.138
G_0 phase	49.658	41.280	37.895	37.055	44.469	40.458	39.580
G_1 phase	25.968	24.578	22.485	22.393	26.134	23.046	22.393
S phase	9.013	15.839	19.787	20.357	17.089	16.691	17.073
G_2 phase	11.251	11.474	11.245	10.563	13.513	12.985	12.458
Mitotic phases	3.125	5.950	7.587	8.958	5.587	5.890	6.120
Proliferation activity	23.389	33.298	38.619	39.878	36.189	35.566	35.651
Non divided cells	76.425	65.717	57.303	57.006	61.603	63.556	61.973

Table 2. Mitotic phases and changes in the cell cycle phases (G_0 , G_1 , S, G_2) in both treated with phosphoren and untreated groups

Doses and gray of cells at different factores	Control	1/2R	R	2R	10 min	1 h	2 h
Nicrotic cells	0.000	1.564	2.012	0.897	0.000	1.658	0.000
G_0 phase	49.658	47.258	40.128	39.085	46.096	44.480	42.789
G_1 phase	25.968	25.158	28.158	27.458	21.394	25.680	26.458
S phase	9.013	10.856	11.895	13.119	11.161	12.146	9.283
G_2 phase	11.251	10.655	11.414	11.742	12.314	11.124	12.216
Mitotic phases	3.125	4.251	5.985	6.897	6.101	4.325	4.562
Proliferation activity	23.389	25.762	29.294	31.758	29.576	27.595	26.061
Non divided cells	76.425	72.850	70.266	67.431	67.490	70.843	70.216

Figs. 3–5. Active (red color) and inactive (yellow color) chromatin in interphase cells of *Vicia faba* root tips. 3) Control. 4) Rhizobacterien biofertilizer treatment with R dose. 5) Phosphren biofertilizer treatment with 1/2R dose. Monitor picture phtograph 10×10×45.

phorelate it, it is converted to an active form. The level of activated MPF varies through the cell cycle peaking during mitosis, (Bruce *et al.* 1994).

Inoculation with the different used biofertilizers increased nitrogen and phosphorus contents in both plants and soil (Abu-Zekry 2000). So, we can suggest that, the tested biofertilizers may provide abundant precursors for DNA and protein biosynthesis. Also, it may play a role in phosphorylation of (MPF).

Furthermore, the effect of biofertilizers might be due to the stimulation effect of cyclin gene transcription before the onset of DNA synthesis as a result of the percentage of growth hormones (Dahl *et al.* 1995). This growth hormones may be produced as metabolites excreted by the biofertilizers (Abu-Zekry 2000).

Table 3. Effect of seed treatments with different doses and time treatment of Rhizobacterien biofertilizer on mitotic index and percentage of abnormalities in roots of *Vicia faba*

Experiment		Total number of cells	Number of dividing cells	Percent of M.I. $\pm$ S.E.	Total % of abnormal cells $\pm$ S.E.	% of lab. interphase
Control		5140	308	6.0 $\pm$ 0.5	5.0 $\pm$ 1.6	0.00
Different doses of Rhizobacterien	1/2R	5250	368	7.0* $\pm$ 6.4	30.0** $\pm$ 1.0	0.40
	R	5228	434	8.3* $\pm$ 0.3	34.0* $\pm$ 4.0	0.38
	2R	5580	530	9.5* $\pm$ 0.6	35.7** $\pm$ 2.4	0.29
Time of treatment with R	10 min	5000	365	7.3* $\pm$ 0.8	23.2* $\pm$ 1.3	0.36
	1 h	4980	393	7.9* $\pm$ 0.8	26.7** $\pm$ 2.3	0.28
	2 h	5380	468	8.7* $\pm$ 2.7	28.9** $\pm$ 1.1	0.48

Table 4. Effect of seed treatments with different doses and time treatment of Phosphoren biofertilizer on mitotic index and percentage of abnormalities in roots of *Vicia faba*

Experiment		Total number of cells	Number of dividing cells	% of M.I. $\pm$ S.E.	Total % of abnormal cells $\pm$ S.E.	% of lab. interphase
Control		5140	308	6.0 $\pm$ 0.5	5.0 $\pm$ 1.6	0.0
Different doses of Phosphoren	1/2R	5827	385	6.6 $\pm$ 0.8	32.7* $\pm$ 4.2	0.5
	R	5050	348	6.9 $\pm$ 0.8	35.0** $\pm$ 1.4	0.43
	2R	5100	418	8.2** $\pm$ 1.6	35.6** $\pm$ 2.7	—
Time of treatment with R	10 min	5310	380	7.2** $\pm$ 1.4	27.6** $\pm$ 2.7	0.39
	1 h	5250	415	7.9* $\pm$ 1.9	29.1** $\pm$ 2.0	0.50
	2 h	4900	326	6.7 $\pm$ 1.0	33.1** $\pm$ 1.8	0.70

* Significant from control at 0.05 level (t-test). ** Highly significant from control at 0.01 level (t-test).

Also, increase in S phase may be due to the hormonal effect of the biofertilizers which stimulate the cell to grow to the maximum size needed for transition from G₁ to S phase or for entry into mitosis *i.e.* stimulate from G₂ to mitosis (Davidson 1991), then lead entry to increase in mitotic activity.

Cytological effects

At the microscopical study, results given in Tables 3, 4 and illustrate the cytological effects of seed treatments with Rhizobacterien and Phosphoren biofertilizers on mitotic activity in the root tip cells of *Vicia faba* compared with control. There was a significant or highly significant increase in the mitotic index, where this increase was dose and duration dependent, and the Rhizobacterien was more effect than Phosphoren.

In general, the 2R dose induced more pronounced effect on mitotic index, compared with the other biofertilizer treatments, its values were recorded 9.5 $\pm$ 0.6 and 8.2 $\pm$ 1.6 after treatment with Rhizobacterien and Phosphoren respectively compared with 6.0 $\pm$ 0.5 of the control. Several investigators reported the same results (Duanchanqun *et al.* 1995, Gabara *et al.* 1995, Hossni and El-tarras 1977, El-Nahas 1999, Shehab *et al.* 2000).

The acceleration of the rate of cell division in *Vicia faba* root cells treated with the biofertilizers might be due to the increased rate of biosynthesis of nucleic acid and other substances required for cell division. El-Awady (1996) studied the effect of N-fertilizer (Urea, ammonium sulphate, calcium nitrate) on root tip of *Allium cepa*, she found that the fertilizer increased the rate of cell division, this increase in mitotic activity was dose dependent.

The cytological examination revealed that the 2 used biofertilizers exert a significant and/or highly significant increase in the percentage of total chromosomal abnormalities in *Vicia faba* root

Figs. 6–9. 6) Chromosome stickiness of Metaphase after treatment with 1/2R for 10 min of Rhizobacterien. 7) Chromatid bridge with stickiness of anaphase after treatment with 2R Phosphoren. 8) Disturbance Metaphase after treatment with R of Rhizobacterien. 9) Polyploidy anaphase after treatment with 2R of Phosphoren.

cells. This increase was dose and time dependent. Also, it can be recognized that Rhizobacterien was less effective in induction of abnormal mitotic division than Phosphoren, but 2R dose was considered the highest values of the percentage of total abnormalities at the 2 biofertilizers and its values were 35.7 ± 2.4 and 35.6 ± 2.8 at Rhizobacterien and Phosphoren respectively compared with control 5.0 ± 1.6 (Tables 3, 4). The 2 used biofertilizers produced similar types of chromosomal abnormalities in mitotic division. Stickiness, spindle disturbance, bridges, laggards, break and fragments, polyploidy, irregular prophase, despiralization and C-metaphase and anaphase were observed.

Chromosomal stickiness was more frequent type of abnormalities recorded in all the mitotic stages (Figs. 6, 7). According to Patil and Bhat (1992) stickiness is a type of adhesion involving mainly the proteinacious matrix of chromatin.

The induction of bridges could be attributed to the stickiness at anaphase (Liu *et al.* 1994), or due to the formation of dicentric chromosome as a results of breakage and reunion (Sahi and Singh 1996).

Disturbance of the chromosomes was observed at a considerable frequency at metaphase and anaphase (Figs. 8, 9). The induction of this type may suggest that the used biofertilizers caused partial disturbance in the spindle apparatus or partial suppression of spindle formation or in accordance with that reported by Pickett-Heaps *et al.* (1982) due to loss of microtubules of the spindle fibers.

The induction of lagging chromosomes and multipolar ana-telophase configurations leads to the separation of unequal number of chromosomes in the daughter nuclei. This is manifested by scoring of fractions of cells with DNA amount $<2C$ value and cells with DNA amount $>4C$ value following treatment with biofertilizers. The percentage of laggards, breaks and bridges indicate the clastogenic effect of the biofertilizers on *Vicia faba* cells.

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