# REPORT

**on the bilateral German-Ukrainian DFG project**

**“Influence of nutrients and stress on the biosynthesis of phenolics (fagopyrin, rutin, hypericin and chlorogenic acid) in buckwheat plants”**

**“Einfluss von Nährstoffversorgung und Stress auf die Biosynthese von phenolischen Substanzen (Fagopyrin, Rutin, Hypericin und Chlorogensäure) in Buchweizen”**

**between**

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## The main results obtained in project

The results obtained during research projects have been published as:
1. Sytar O., Kosyan A., Taran N., Smetanska I. 2014. Antocyanins as marker for selection of buckwheat plants with high rutin content. Gesunde Pflanzen 66:165–169, Impact Factor 0.45
2. Sytar O., Borankulova A., Hemmerich I., Rauh C., Smetanska I. 2014. Effect of chlorocholine chlorid on phenolic acids accumulation and polyphenols formation of buckwheat plants. Biological Research 2014, 47:19 doi:10.1186/0717-6287-47-19, Impact Factor 1.04
3. Smetanska I., Sytar O. 2015. The contribution of buckwheat genetic resources to health and dietary diversity. J Biotechnol Biomater 2015, 5:2, 95. DOI: 10.4172/2155-952X.S1.038 IF 1.94

The results of experiments have also been presented:
1. at conference of Hellenic Society of Biologistsin in Volos Greece on 21-23th May 2015
Sytar O., Smetanska I. Investigation of the mechanism of action(s) of fagopyrin and hypericin isolated from buckwheat in yeast model system
2. at the conference 8th Euro Biotechnology Congress on August 18-20, 2015 in Frankfurt
Sytar O., Smetanska I. The contribution of buckwheat genetic resources to health and dietary diversity

Part 1:
**The effect of chlorocholine chlorid on accumulation of phenolic acids and polyphenols in buckwheat plants**

**Background:** The effect of chlorocholine chloride (CCC) on phenolic acids and polyphenols accumulation in different anatomical parts (stems, leaves, and inflorescences) of common buckwheat (*Fagopyrum esculentum* Moench) in the early stages of vegetation period was investigated.

**Results**: Treatment of buckwheat seeds with 2% of CCC has increased the content of total phenolics in stems, leaves and inflorescences (Fig.1).

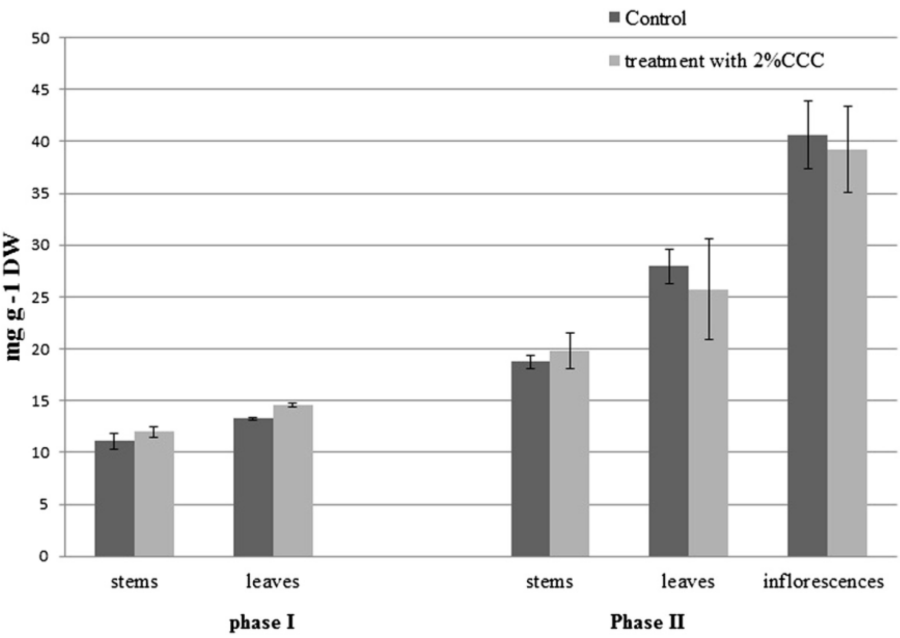



**Figure 1.** Total phenolics content in buckwheat plants at the phase I (formation of buds) and phase II (beginning of flowering), non-treated and treated with 2% CCC.

By the analyzing of different anatomical parts of buckwheat plants, 9 phenolic acids as vanilic, ferulic, trans-ferulic, chlorogenic, salycilic, cinamic, p-coumaric, p-anisic, methoxycinamic as well as catechins were identified. The levels of phenolic acids varied significantly not only among the plant organs, but also during the different stages of vegetation period. The changes in contents of chlorogenic, ferulic, and trans-ferulic acids correlated with the content of salycilic acid. The content of these phenolic acids has been significally increased after 2% CCC treatment at the phase I (formation of buds) in stems and at the phase II (beginning of flowering) in leaves and inflorescences.

The content of catechins has been increased at the early stages of vegetation period after treatment with 2% CCC (Figure 2).

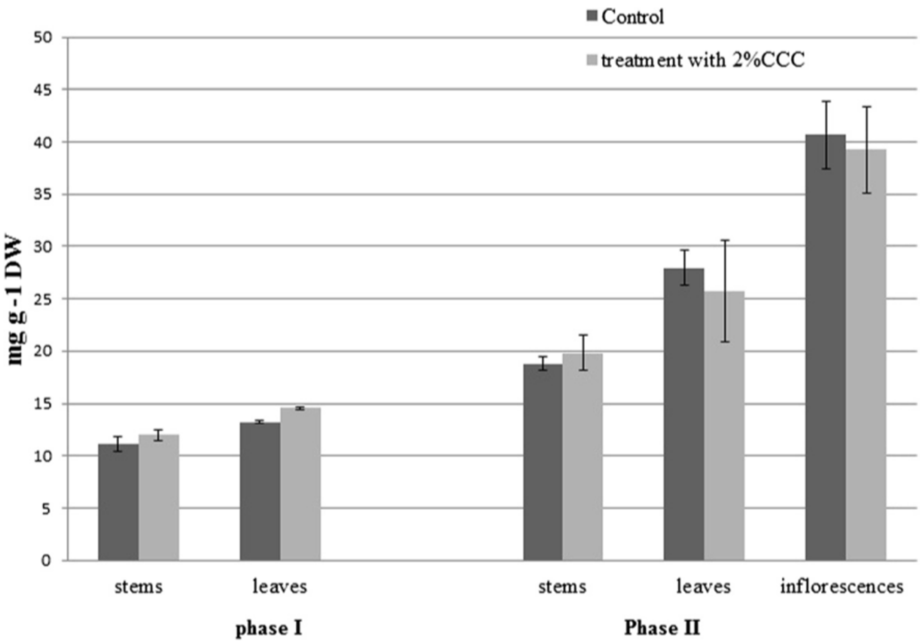



**Figure 2.** Content of catechins in different parts of buckwheat plants after the treatment with 2% CCC.

**Conclusions**: The vegetative parts of buckwheat plants as leaves, stems, and inflorescences contain higher level of antioxidants as buckwheat seeds. However, the vegetative mass of buckwheat plants is nowadays not yet used in food industry. The topic of this study was to measure the dynamic of antioxidants in vegetative parts of buckwheat plants. We also treated plants with chlorocholine chloride, because this substance is known to increase content of phenolic compounds. The obtained results shown that influence of CCC on the content of individual phenolics can be a result of various mechanisms of CCC uptake, transforming, and translocation in plants. The level of identified phenolic acids was significantly influenced by CCC not only among the plant organs, but also between early stages of vegetation period.

**Part 2:**
**Anthocyanin's as marker for selection of buckwheat plants with high rutin (P-vitamin, rutinoside) content**

The use of bioactive substances in food and pharmaceutical industries is limited due to lack of information about their biological activity and limited number source of raw materials. The search for efficient and economically profitable sources of flavonoids with P-vitamin activity is one of the major concerns of the pharmaceutical industry (Hollman and Katan 1999).

Rutin is one of the phenolic compounds presented in buckwheat plants (Park et al. 2000). Rutin content in vegetative mass of buckwheat (leaves, inflorescences and stem) ranges from 2 up to 9 % (Park et al. 2000; Kreft and Fabjan 2006), which may vary according to growth location and cultivar. It has been reported that groats and hulls of buckwheat contained approximately 0.20 mg $g^{-1}$ and 0.84–4.41 mg $g^{-1}$ rutin, respectively (Oomah and Mazza, 1996). Tartary buckwheat is an excellent source of rutin because the groats of Tartary buckwheat contain 80.94 mg $g^{-1}$ rutin. The content rutin in flowers of Tartary buckwheat in full bloom stage reaches 6.8 %, in leaves 5.5 %, and in stalks 3 %.

**Results:** This study was focused on the selection of buckwheat species and cultivars, such as *Fagopyrum esculentum* Moench (Cultivars Lileya, Bilshovik, Rubra), *F. tataricum* G (ssp. Rotundatum (Bab) Krot. and ssp. tuberculatum Krot.), *F. cymosum* Meissn, and *Fagopyrum giganteum* Krot with high rutin content. In Rubra cultivar rutin in vegetative organs of plants correlated with anthocyanin content (Table 1).

**Table 1.** Rutin content in vegetative organs of buckwheat in the full-bloom stage, % dry weight (DW)

| Species | Stem | Leaves | Inflorescences |
|---|---|---|---|
| *F. esculentum*, (Lileya) | 0,7 | 5,5 | 6,5 |
| *F. esculentum*, (Rubra) | 1,3 | 8,0 | 13,4 |
| *F. esculentum*, (Bilshovik) | 0,7 | 5,9 | 6,8 |
| *F. tataricum* v. rotundatum | 1,0 | 5,3 | 6,1 |
| *F. tataricum* v. tuberculatum | 0,7 | 4,6 | 4,8 |
| *F. cimosum* | 1,1 | 2,5 | 6,4 |
| *F. giganteum* | 1,0 | 2,8 | 6,6 |
| $LSD_{05}$ | 0,15 | 0,32 | 0,41 |

The presence of anthocyanins in the vegetative organs of buckwheat can be a reliable genetic marker for screening plants with high content of rutin (Figure 3).

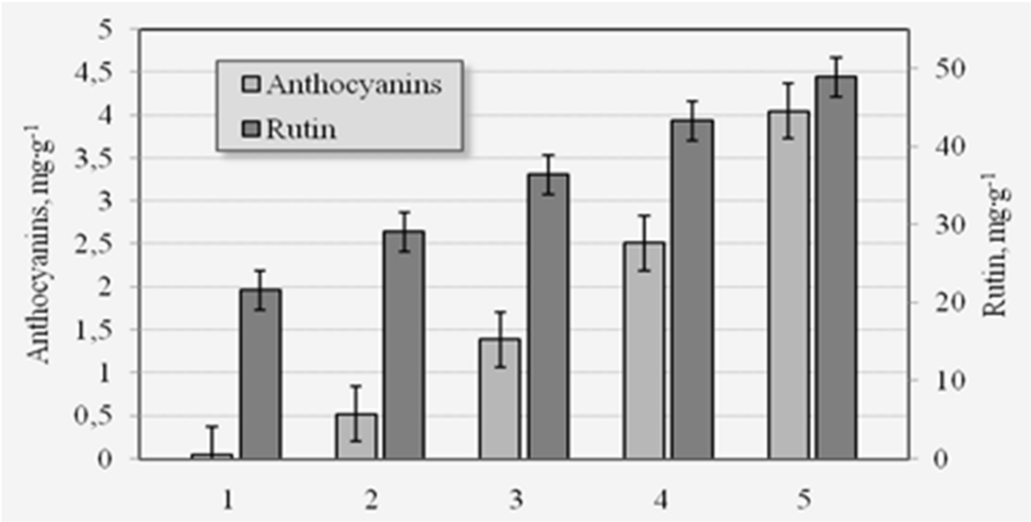



**Figure 3.** The content of rutin und anthocyanins in leaves of buckwheat seedlings.

For definition of correlation between the anthocyanins and rutin content in the vegetative organs of buckwheat several series of experiments have been planned. For analysing we have used seedlings of buckwheat in the early development stages (cotyledon and 2nd true leaf). All plants were divided into five groups according to the spreading of red color (as visible indicator for anthocyanins):

1. the red color () was absent on the surface of leaves of a seedlings,
2. lower leaf surface was partially red,
3. lower leaf surface with red,
4. lower leaf surface was completely red and the upper surface was partially red,
5. lower surface and upper surface of leaves was red.

In the third generation of selection process with the proposed selection method, a genetic line of Rubra cultivar with high rutin content in the vegetative mass has been obtained during our experiments. The proposed method of selection is based on the color visual assessment of plant parts. The red color of vegetative organs of buckwheat plants can be a marker for selection of buckwheat cultivars with high anthocyanin and rutin content.

**Part 3: Investigation of the mechanism of action(s) of fagopyrin and hypericin, isolated from buckwheat plants on yeast model system** (the results are in preparation for publishing)

Buckwheat (*Fagopyrum esculentum* Moench) extract contains high amount of phenolic compounds possessing anti-proliferative activity. However, for the better understanding of this phenomenon, it is necessary to investigate the factors and mechanisms, responsible for this functioning. In the present study, we used the budding yeast *Saccharomyces cerevisiae* as model organism to study the effect of two buckwheat phytochemicals on eukaryotic cell cycle and morphogenesis.

Using flow cytometry (FC) combined with microscopy we have shown that the exposure of yeast cell to the different doses of pure fagopyrin and hypericin resulted in changing of its cell cycle progression and morphology. FC analysis gave the results about yeast cell viability with relative quantification of dose-dependent toxicity and cell proliferation. Specifically, our results have shown that fagopyrin and the closely related hypericin caused minor toxicities and had similar, but not identical effect on cells. These results were verified by microscopic observations which provided additional information on aberrant cell morphologies. The combined analysis of cells by flow cytometry and microscopy revealed previously unsuspected effect of fagopyrin and hypericin on eukaryotic cells.

**Methods**

A cycling yeast culture at mid-log phase was splited in three subcultures and each one of them was treated with

i) Fagopyrin (4%),
ii) Hypericin (4%) and
iii) YPD (Control).

The cells were cultivated at 30 $^{0}$C for 2h. Subsequently, the cells were spun down, washed in 1X PBS, and fixed in 70% ethanol. For microscopic observations of the cells a Zeiss Axioscope 40 was used with 100X lenses. At least one hundred cells were observed per treatment. Furthermore, identical samples were analyzed by BD FACS Calibur Flow Cytometer. Cell viability was assessed using fluorescent exclusion dye. In brief, the cells were washed with 1X PBS and stained with BD Horizon Fixable Viability Stain FVS 660 (BD Biosciences) for 10min. The excess stain was washed off with 1X PBS. The cells were initially gated according to forward scatter/side scatter; secondary gates were set on the basis of cell viability. Data analysis was performed using the Flowing Software Version 2.5.

**Results:** Microscopic examination of the cells revealed morphological alterations after two hours of 4% Fagopyrin treatment (F4). Specifically, the cells treated with F4 appeared larger in size compared to the Wild Type ones, and defected in cell division cycle. Although mother and daughter cells didn’t separate, they initiated buds at places different than the default ones. Single cells and groups of several cells were the most prominent phenotypes detected in F4. An even greater enlargement of the cells was observed under 4% Hypericin treatment (H4), indicating that the cells delayed cell cycle progression for longer time compared to F4 treated ones (Figure 4, 5, 6).

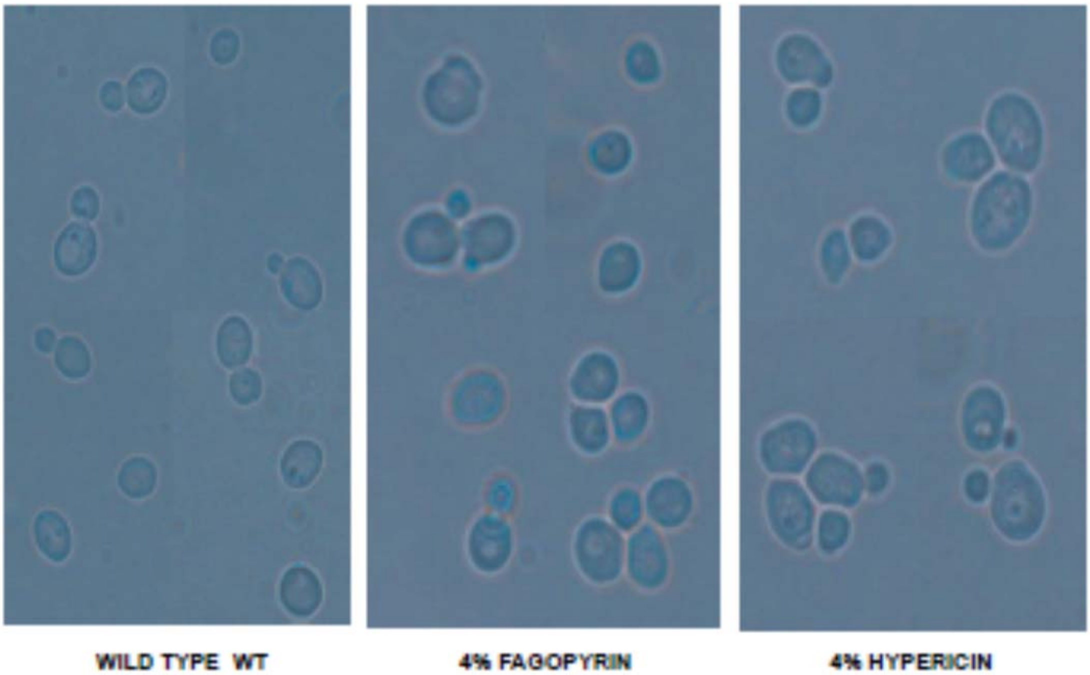



**Figure 4.** Cell phenotypes under Fagopyrin and Hypericin treatment. Microscopic images taken with 100x linses

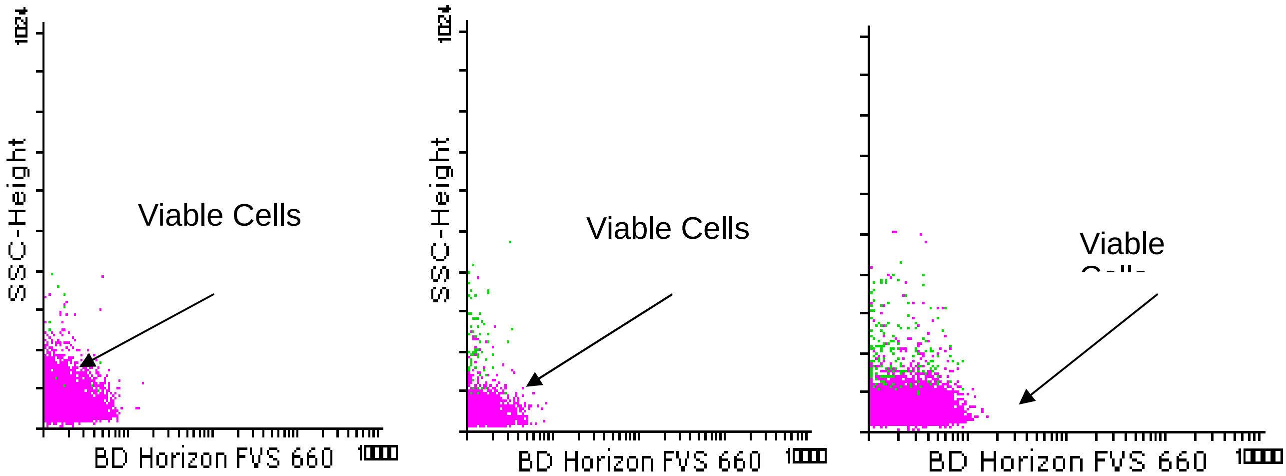
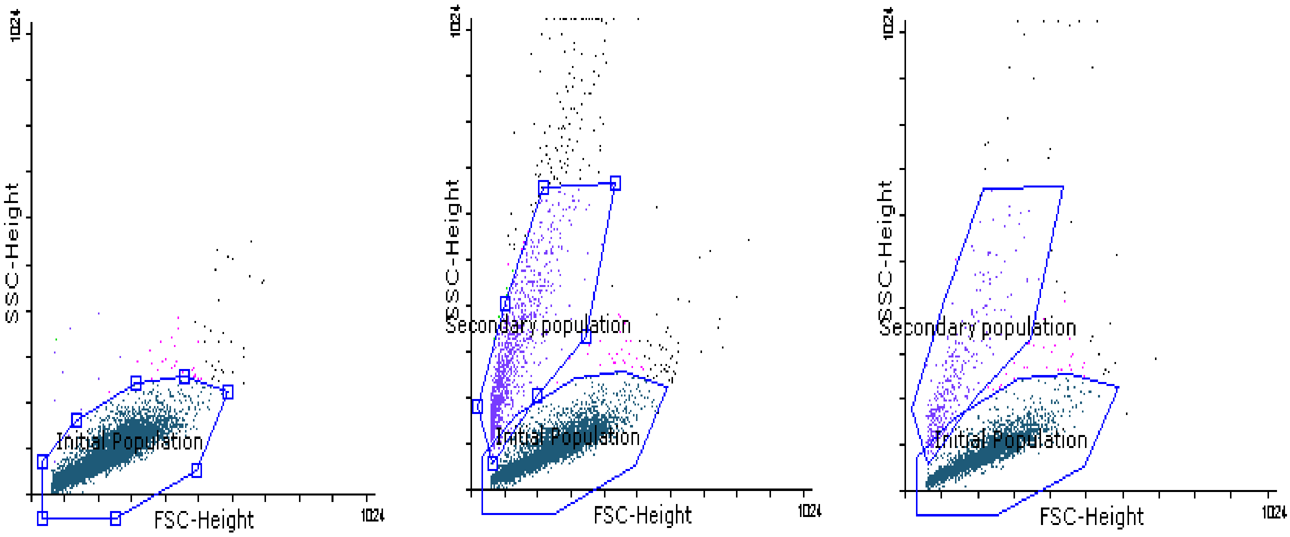



**Figure 5.** The viability of the cells is not affected by the F4 and H4 treatment.



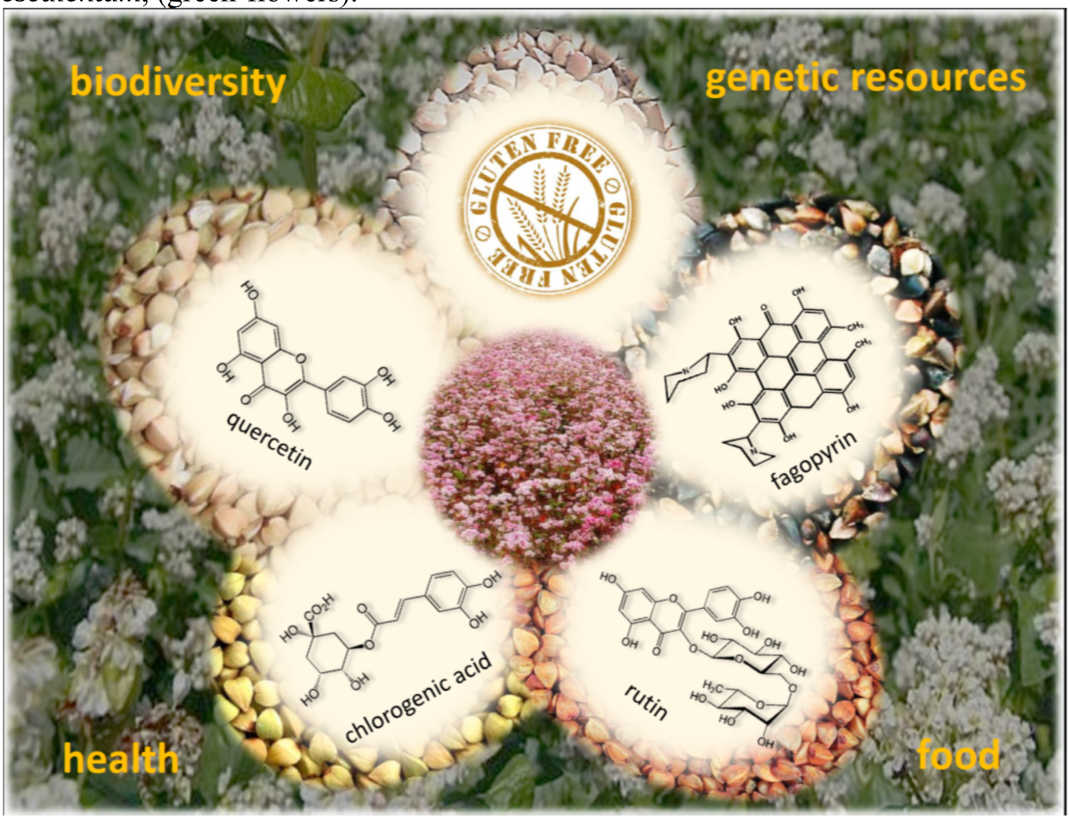

**Untreated Control** **4% Fagopyrin treated** **4% Hypericin treated**

**Figure 6.** The morphology of the cells changed due to the treatment and a new group of small cells with dense structure emerged.

**Conclusions:** Each one of the phytochemicals perturbed the normal cell cycle progression. The differences in the effects of the two substances were subtle. Unsuccessful cell separation at mitosis and initiation of a new budding cycle resulted in the formation of cell aggregates. Based on FACS Analysis the two compounds didn't cause any toxicity to the cells during the 2 hour treatment at the given concentration. Consistent with the microscopic observations FACS Analysis classified the cells under treatment in two categories: *a*) small with dense structure and *b*) large with low degree of complexity. The unexpected findings in yeast may be applicable in other eukaryotic cells and speed up the development of novel antifungal drugs.

**Part 4: Contribution of buckwheat genetic resources to health and dietary diversity**

Despite several reports on the beneficial effects of buckwheat in prevention of human diseases, little attention has been devoted to the variability of biochemical and physiological traits in different buckwheat genetic resources. The biochemical evaluation of buckwheat genetic resources and the identification of the elite genotypes for plant breeding and exploitation have been studied. Various types of bioactive compounds in different varieties provide basic background information needed for the efficient production of buckwheat food with added values. Results of our research work show that presence of anthocyanins in the vegetative organs of buckwheat can be a reliable genetic marker for screening plants with high content of rutin. We have compared *F. esculentum* Moench (cultivars Lileya, Bilshovik, Rubra), *F. tataricum* (ssp. rotundatum Krot. and ssp. tuberculatum Krot.), *F. cymosum* Meissn, and *F. giganteum* Krot. The highest rutin content was measured in inflorescences of *F. esculentum* Rubra. The comparative analysis of total phenolics and phenolic acid composition together with antioxidant activities in inflorescences of *F. esculentum, F. tataricum rotundatum* and *F. esculentum* (green-flowers) shown that *F. esculentum* inflorescences have been characterized by the highest content of salicylic acid (115 mg 100 g-1 DW) and methoxycinnamic acid (74 mg 100 g-1 DW). The highest content among the investigated buckwheat inflorescences of vanillic acid, trans-ferulic acid, chlorogenic, acid and p-anisic acid was found in the *F. tataricum*. Inflorescences of *F. esculentum* (green-flowers) have a high content of chlorogenic acid (16 mg 100 g-1 DW), p-anisic acid (872 mg 100 g-1 DW). The highest fagopiryn content was measured in inflorescences of *F. esculentum*, (green-flowers).




**Graphical abstract of results obtained in frames of the project.**

**References**:

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