Evaluation of the antigenotoxic potential of fresh bovine whey in onion meristematic roots exposed to Quizalofop-P-tefuryl

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Abstract. Whey is a protein complex derived from milk, being a functional food with multiple health benefits. In this paper, the antigenotoxic potential of fresh bovine whey (FBW) in onion (*Allium cepa*) meristematic roots exposed to Quizalofop-P-tefuryl (QPt) herbicide was evaluated using the *Allium* assay. Firstly, the *Allium cepa* meristematic roots obtained after a short germination of 24 hours in distilled water were subjected to a pre-treatment with FBW in three different concentrations (500, 1000 and 2000 µl/L) for 24 hours. After that, there was a post-treatment with QPt herbicide (100, 500 and 1000 µl/L), for another 24 hours. All variants were tested alongside a negative control (onion root tips in distilled water) and a positive control (onion root tips treated with 500 µl/L QPt).

The genotoxic effects of QPt were observed in all treatment variants, through the low rate of mitosis and through the induction of a large number of chromosomal and nuclear abnormalities (bridges, laggards, rings and strap nuclei). On the other hand, the fresh bovine whey improved the mitotic activity and reduced the index of chromosomal aberrations in variable percentages, in all treatment variants. These results suggest the cytoprotective potential of FBW against the cytotoxic and genotoxic effects of the tested herbicide. Although the mechanism of antigenotoxicity is unknown, it seems plausible that the whey protein acts as a blocking agent by chemical or physical interaction with the QPt components. Nevertheless, additional studies are needed to determine with certainty this potential.

Keywords: allium assay, herbicide, genotoxic, whey, antigenotoxic

INTRODUCTION

Genotoxicity is the ability of various agents to cause damage to genetic material and to affects cellular components involved in the functionality and behavior of chromosomes (Bhattachar, 2011; Nagarathna et al. 2013). Agents able of causing genetic toxicity are described as genotoxic and are called genotoxins. One of the categories of genotoxins that cause damage to genetic material is pesticides (Anguiano-Vega et al. 2020; Kim et al. 2017).

Higher plants are used in many experiments as indicator plants that show the cytogenotoxic effects of chemicals that pollute the environment (Yıldız et al. 2009; Bonciu et al. 2018; Deveci Özkan et al. 2019). In this context, *Allium cepa* L. is a widely used indicator plant for testing the genotoxic/antigenotoxic potential of various chemicals (Bonciu et al., 2018; Datta et al., 2018; Khanna and Sharma, 2013).

Pesticides (fungicides, insecticides and herbicides) are chemicals used against pests and weeds in agriculture. Certain pesticides cannot be broken down by microorganisms or the human body's enzymatic equipment, and they can accumulate. For this reason, pesticides represent a source of toxic risk, due to their persistence in soil (Datta et al. 2018; Srimurali et al. 2015), plants (Deveci Özkan et al. 2019; Roșculete et al. 2019) and in the human body (Andersson et al. 2021; Hernandez et al. 2016; Van Maele-Fabry et al. 2019; Yusa et al. 2015).

Keeping weeds under control is a constant practice of modern agriculture to ensure high yields by suppressing the growth of unwanted wild species that compete for the same resources with agricultural plants (Bartucca et al. 2019; Loddo et al. 2020). But, the frequent and excessive use of these chemicals has been identified as a serious threat to the environment and human health (Parveen et al. 2016).

Quizalofop-P-tefuryl (QPt) is a post-emergence herbicide used for the control of the grass weeds in agricultural and horticultural crops (potato, sugar beet, sunflower, oilseed rape, peanut, etc.). The active ingredient is rapidly adsorbed by the leaves of grass weeds, producing their well-wishing and consequent death. Its mode of action involves the inhibition of acetyl CoA carboxylase activity.

Whey is full of cysteine, a substance necessary for the production of glutathione, a powerful antioxidant that protects the body from infections and toxins (Gupta et al. 2017). Thus, whey helps strengthen the immune system. Glutathione is also quite effective in the treatment of the thyroid gland, cancer, sclerosis and Parkinson's disease (Marshall et al. 2004).

Current studies suggest that the fresh bovine whey (FBW) is much more than a protein source with a particularly nutritious composition of essential amino acids (Jaladat et al. 2022; Park et al. 2021; Walzem et al. 2002). Thus, FBW is a real complex cocktail, which contains, in addition to proteins, peptides, complex lipids and oligosaccharides; all of these substances act as a growth factors, toxin binding factors, antimicrobial peptides, prebiotics and immune regulatory factors in mammals (Pan et al. 2013; Teixeira et al. 2019).

Consumption of whey products can modulate redox biomarkers to reduce oxidative stress (Giblin et al. 2019). This bioactivity has been partially attributed to the whey peptides using a series of biochemical or cellular *in vitro* assays (Abdel-Wahhab et al. 2013; Corrochano et al. 2019; Garg et al. 2018; Falkowski et al. 2018).

A number of studies have demonstrated the antioxidant bioactivity of whey products and increasing glutathione levels (Brandelli et al. 2015; Yadav et al. 2015; Zhang et al. 2012). Glutathione (tripeptide with an important antioxidant role in the body of plants, animals, fungi and bacteria) prevents the destruction of some cellular components and detoxifies various endogenous and exogenous toxins (Kasperczyk et al. 2013; Pizzorno et al. 2014). Some cellular lines exposed to various whey products showed increases in glutathione levels, with some exceptions (Corrochano et al. 2019).

On the other hand, some studies suggest the adverse effects of FBW when it is consumed in excess (Amanzadeh et al. 2003; Vasconcelos et al. 2021). Practically, 20-25 grams of whey protein powder can be consumed every day, depending on the active or sedentary lifestyle. Whey is contraindicated for people allergic to dairy products and the high consumption of whey protein can lead to an increase in the percentage of body fat and stress on the kidneys, an increase in cardiovascular and osteoporosis risks, the appearance of nausea, headaches, cramps, reduced appetite, etc. (Aparicio et al. 2011; Aydın et al. 2018; Hattori et al. 2017; Vasconcelos et al. 2021).

FBW (pH < 5.1) as a by-product from the manufacture of hard, semi-hard or soft cheese and rennet casein is known as sweet whey. The main constituents of FBW are shown in Table 1 (Dinkci, 2021).

The antigenotoxic potential of whey proteins in the field of medicine was suggested by many results (Aydın et al., 2018; Jaladat et al., 2022; Marshall, 2004; Park et al., 2021; Teixeira et al., 2019). On the other hand, in the specialized literature there is a lack of results regarding the antigenotoxic potential of FBW following plants exposure to various chemical substances, such as pesticides. In this context, we initiated this study for evaluation of the antigenotoxic potential of FBW in onion (*A. cepa*) meristematic root tips exposed to QPt herbicide.

MATERIALS AND METHODS

Plant material

In this study, a number of ten onion (*A. cepa*, 2n=16) bulbs (procured from a local vendor) were used as biological material for each treatment variant. The outer scales were

removed, and older dry roots were scrapped off in order to promote the emergence of new roots. QPt is the active substance of Pantera herbicide (producer Chemtura S.R.L. Italy). This was purchased from a local specialty store for phytopharmaceutical products and was used as the test substance. The onion bulbs were immersed in glasses with distilled water for a short germination (24 h) and then were subjected to a pre-treatment with FBW in three different concentrations (500, 1000 and 2000 µl/L) for 24 hours. After that, there was a post-treatment with QPt herbicide (100, 500 and 1000 µl/L), for another 24 hours. The QPt herbicide concentrations were established according to the dose recommended in agricultural practice. The concentrations of FBW were randomly set, because in the literature there is no data related to the testing of FBW in plants, but only some results on animals.

The length of the meristematic roots was measured and recorded after each treatment stage as roots length average (RLA). Likewise, microscopic analyses were performed after each treatment stage and for each sample, in order to determine the mitotic index (MI), the indices of mitosis phases (IP=prophase index; IM=metaphase index; IA=anaphase index; IT=telophase index) and to identify the chromosomal aberrations.

For this study, the variants were tested alongside a negative control (onion root tips in distilled water) and a positive control (onion root tips treated with 500 µl/L QPt herbicide). The experiment was performed in laboratory, at room temperature (24±2°C).

Microscopic preparations

After measuring and recording the root growth following germination, pre-treatment with FBW and respectively post-treatment with QPt, the biological material was prepared for the microscopic stage. Thus, *A. cepa* roots were fixed in ethanol: acetic acid (3:1) and hydrolysed in 1N hydrochloric acid (HCl) at 60ºC for 5 min. Roots with a length of approximately 1 cm were stained through immersion in 3-5 ml Schiff reagent (30 minutes) and then transferred on clean slide and crushed in drop of 2% acetocarmine. The microscopic preparations were performed by squash technique.

All slides were labelled before microscopic analysis. Five random microscopic fields from each slide were scored.

The viewing microscopic area was divided into three viewing sections and then, in each viewing section, the cells were counted and recorded in prophase, metaphase, anaphase and telophase. The MI and mitosis phase index were calculated using the following formulas:

MI (%) = × 100

IP (%) = × 100

IM (%) = × 100

IA (%) = × 100

IT (%) = × 100

The index of the total abnormalities (ITA) was also calculated:

ITA (%) = × 100

Chromosomal aberrations and nuclear anomalies were determined by scoring cells with bridges, laggards, rings and strap nucleus in randomly picked three zones per slide. Photomicrographs of cells showing mitosis, chromosomal aberrations and nuclear anomalies were taken using the digital microscope Optika B-190TB (Optika, Italy), 1000x magnification.

Statistical analyses

The experiment was organized according to a randomized complete design with three replications and minimum 1000 cells were analysed. Statistical analysis was done using MS Excel 2016. The data obtained were analysed by one-way analysis of variance (ANOVA) and Duncan's multiple range test by using statistical software SPSS version 20 for Windows. Significance was considered at $p < 0.05$. Data were expressed as mean ± standard error (SE) (Gomez and Gomez, 1984). The experiment was conducted in triplicate and minimum 1000 cells were analysed for each sample.

RESULTS

The length of the meristematic roots was measured and recorded after each treatment stage (Figure 1). Thus, after 24 hours germination in distilled water, RLA recorded values ranged between 0.3 and 0.6 cm, while the value of the negative control was 0.5 cm. After pre-treatment with FBW, the highest RLA value was found in sample V2 (1000 µl/L) - 1.9 cm, followed by V1 (500 µl/L) - 1.7 cm and V3 (2000 µl/L) - 1.6 cm. Thus, a more intense growth of onion roots is found in all variants, compared to the negative control, in variable percentages between 58.3-33.3%. On the other hand, after post-treatment with QPt herbicide, the highest RLA value was found in variant V2 (500 µl/L) – 2.3 cm, followed by V1 (100 µl/L) – 2.1 cm and V3 (1000 µl/L) - 1.7 cm. Thus, a more intense growth of onion roots is found in V2 and V1 samples, compared to the negative control, in variable percentages between 27.7-16.6%. In the case of V3 sample, the RLA value was 5.5% lower than the negative control.

Table 2 presents the results of the influence of FBW and QPt on the MI and mitosis stages index in *A. cepa* root tips.

It was found that, following pre-treatment with FBW, the MI in two variants, namely V1 (62.6%), respectively V2 (65.3%), was higher compared to the negative control (54.2%). After the treatment with 2000 µl/L FBW (V3), the MI value was lower (51.4%) than the value recorded for the negative control. On the other hand, higher values of MI were found in all variants compared to the positive control.

Regarding the post-treatment with the QPt herbicide, an increased MI was recorded in the same variants, namely V1 (66.2%) and V2 (71.5%), compared to the negative control. The treatment with 1000 µl/L QPt (V3) induced a decrease of MI compared to the value recorded by the negative control, but higher than the value recorded by the positive control. It can be appreciated that the 2000µl/L FBW and 1000µl/L QPt concentrations induced a mitodepressive effect in meristematic root cells of *A. cepa* in a time and concentration-dependent manner.

The indices of mitosis phases had different values compared to the control variant, between 78.7-88.4% IP, 3.0-5.5% IM, 3.1-5.1% IA and 5.5-10.7% IT, respectively.

The results regarding chromosomal and nuclear abnormalities induced by FBW and QPt in *A. cepa* root tips are presented in Table 3.

In all treatment variants, the appearance of a variable number of chromosomal aberrations and nuclear anomalies was observed, the most important being the following: bridges, laggards, rings and strap nuclei (Figure 2). Thus, the bridges were observed with a frequency ranged between 2.0-3.4% after the treatment with FBW and an increased frequency of 2.4-4.1% after the treatment with QPt, respectively. Laggards had values between 4.2-5.1% after the treatment with FBW and respectively 4.0-5.4% in the case of treatment with QPt. Also, cells with ring chromosomes had values between 1.5-2.2% in the case of treatment with FBW and respectively 1.9-2.2% in the case of treatment with QPt. On the other hand, the nuclear abnormalities of the strap nucleus type were recorded with a frequency of 2.2-3.9% and 3.0-5.3%, after the treatment with FBW and QPt, respectively. It was found that all these abnormalities recorded higher values than the negative control but much lower than the positive control. A similar evolution of the records was determined for the index of the total abnormalities (ITA%), that ranged between 9.9-14.6% (after FBW treatment) and 11.3-16.9% (after QPt treatment).

DISCUSSION

In the last years, many researchers have found novel bioactive phytocompounds able to counteract the effects of some physical and chemical mutagens that can affect the health of humans, animals and the environment (Bonciu et al., 2018; Dimitrov et al., 2006; Franco-Ramos et al, 2020). The ability of a substance to cause cytotoxicity is measured by its capability to decrease cell proliferation (Franco-Ramos, 2020).

The challenge of identifying and developing of some therapies through plants help or some dietary strategies represents a major public health challenge. Thus, several studies have shown the antigenotoxic potential of different plants (López-Romero et al., 2018; Park et al., 2018; Stavric et al., 1996). Also, whey protein supplementation is a dietary strategy widely used in the field of oncology (Abdel-Wahhab et al., 2013; Gupta and Prakash, 2017; Teixeira et al., 2019). This emerging dietary strategy harbouring several benefits translated well into animal models of cancer and in humans. At the molecular level, whey protein subfractions display appealing anti-cancer effects (Teixeira et al., 2019).

In order to highlight the cytological activity and the occurrence of some cytogenetic abnormalities induced to QPt in plant meristematic roots it was chosen *Allium* test because is one of the simplest, inexpensive and valuable tests for determining the cytotoxicity of chemical substances on plants. As Shetty et al. (2017) states, *A. cepa* root chromosomal aberration assay was chosen as it offers an easily adaptable method for screening mitotic/genotoxic/antigenotoxic activity of any bioactive chemical.

In our study, in all QPt herbicide treatment variants, the appearance of a variable number of chromosomal aberrations and nuclear anomalies was observed, the most important being bridges, laggards, rings and strap nuclei. The results obtained showed the strong cytotoxic effect of QPt herbicide, by reducing the MI in onion roots. Thus, it can be appreciated that the 1000 µl/L QPt induced a mitodepressive effect in meristematic root cells of *A. cepa* in a time and concentration-dependent manner.

In this context, there are many results regarding the cytogenotoxic effects of herbicides on plants or animals. Thus, the results obtained by Dimitrov et al. (2006) show that the tested herbicide (Stomp) did not induce chromosomal aberrations in plant cells of *Crepis capillaris* L., instead it was increased their incidence in mouse bone marrow cells. In the same study on the other hand, the herbicide increased the frequency of micronuclei in both test systems. The authors suggest that the induction of some nuclear and chromosomal aberrations in plant cells may have been due to the herbicide's disruptive effect on the spindle, as all herbicide concentrations produced C-mitosis. Also, the

increased frequency of chromosomal aberrations in mouse bone marrow cells may be due to the biosynthesis of genotoxic metabolites (Dimitrov et al. 2006).

Some dairy products (milk and yogurt) supplemented with red ginseng extract have been shown to have antioxidant and antigenotoxic effects (Park et al. 2018). Also, some studies have shown the potential of lactobacilli and bifidobacteria in dairy products to inhibit the genotoxic activity of chemical compounds (Burns et al. 2000; Lopitz-Otsoa et al. 2006; Tavan et al. 2002).

Our results showed that FBW has improved mitotic activity and reduced chromosomal abnormalities in the meristematic cells of *A. cepa* induced by QPt, a fact that suggests the antigenotoxic potential of FBW. The FBW reduced the aneugenic and clastogenic effects of QPt in *A. cepa* cells. Although the mechanism of antigenotoxicity is unknown, it seems plausible that the whey protein acts as a blocking agent by chemical or physical interaction with the QPt components. However, the continuation of studies in this direction remains open in order to establish with certainty the antigenotoxic potential of FBW in plant cells.

CONCLUSIONS

The experimental results from the present study show that the application of QPt herbicide produced some cytotoxic and genotoxic effects on the meristematic cells of *A. cepa*. These toxic effects increased significantly at dose dependently, but application of FBW ameliorated these negative effects which arise under herbicide stress. Thus, FBW has improved mitotic activity and reduced chromosomal abnormalities in the meristematic cells of *A. cepa* induced by QPt herbicide, a fact that suggests the antigenotoxic potential of FBW. We can conclude that FBW can be able to ameliorate the abnormalities caused in *A. cepa* cells due to herbicides stress. Also, our results suggested that, although the mechanism of antigenotoxicity is unknown, it seems plausible that the whey protein acts as a blocking agent by chemical or physical interaction with the QPt components. Nevertheless, additional studies are needed to determine with certainty this potential.

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Table 1. The main constituents of FBW*

Constituents	%
Total solids	6.00-6.50
Water	93.00-94.00
Fat	0.05
Protein	0.60-0.65
NPN (non-protein nitrogen)	0.20

Lactose	4.50
Ash (minerals)	0.50
Calcium	0.03
Phosphorus	0.04
Sodium	0.04
Potassium	0.14
Chloride	0.09
Lactic acid	0.05

*Source: Dinkci, 2021

Table 2. Results of the influence of FBW and QPt on the MI and mitosis stages index in *A. cepa* meristematic roots*

Specification	Variant/Conc. (µl/L)	MI±SE (%)	IP±SE (%)	IM±SE (%)	IA±SE (%)	IT±SE (%)
NC	-	54.2 ±1.8^{a}	82.9±0.2^{a}	3.9±0.2^{a}	5.5±0.3^{a}	7.7±0.5^{a}
PC	-	33.2 ±1.8^{b}	85.1±0.4^{a}	2.1±0.1^{a}	3.1±0.3^{a}	9.7±0.6^{a}
FBW	V1/500/24	62.6 ± 2.1^{b}	84.2±0.5^{a}	3.5±0.4^{a}	4.2±0.5^{b}	8.1±0.5^{a}
	V2/1000/24	65.3 ±2.9^{b}	83.5±0.9^{a}	3.4±0.2^{a}	3.2±0.1^{c}	9.9±0.7^{a}
	V3/2000/24	51.4 ± 3.1^{a}	85.6±0.7^{b}	3.2±0.2^{a}	4.1±0.5^{b}	7.1±0.4^{a}
QPt	V1/100/24	66.2 ± 2.4^{b}	80.3±0.4^{a}	4.7±0.5^{b}	4.4±0.2^{b}	10.6±0.5^{b}
	V2/500/24	71.5 ± 4.8^{c}	78.7±0.8^{a}	5.5±0.2^{b}	5.1±0.3^{a}	10.7±0.5^{b}
	V3/1000/24	42.7 ± 1.9^{d}	88.4±0.9^{c}	3.0±0.3^{a}	3.1±0.1^{c}	5.5±0.2^{c}

*Means with the same letter in the same column for each application time do not differ statistically at the level of 0.05. NC=negative control; PC=positive control; MI=mitotic index; SE=standard error; IP=prophase index; IM=metaphase index; IA=anaphase index; IT=telophase index. Data are means ± SE of three replicates.

Table 3. Results regarding the chromosomal and nuclear abnormalities induced by FBW and QPt in *A. cepa* root tips*

Specification	Variant/Concentration (µl/L)/Exposure time (h)	B (%)	L (%)	R (%)	SN (%)	ITA±SE (%)
NC	-	0.2	0.8	0.1	1.2	2.3±0.5^{a}
PC	-	4.8	6.2	2.7	7.4	21.1±1.1^{b}
FBW	V1/500/24	2.0	4.2	1.5	2.2	9.9±0.4^{a}
	V2/1000/24	2.7	4.5	1.9	3.1	12.2±0.7^{a}
	V3/2000/24	3.4	5.1	2.2	3.9	14.6±0.6^{a}
QPt	V1/100/24	2.4	4.0	1.9	3.0	11.3±0.4^{a}
	V2/500/24	3.5	4.1	2.2	4.3	14.1±0.4^{b}
	V3/1000/24	4.1	5.4	2.1	5.3	16.9±0.9^{c}

*Means with the same letter in the same column for each application time do not differ statistically at the level of 0.05. NC=negative control; PC=positive control; B=bridges; L=laggards; R=rings; SN=strap nucleus; ITA= index of the total abnormalities; SE=standard error.

Figure 1. *A. cepa* roots lenght average (RLA) (cm) after a short germination of 24 hours in distilled water, followed by pre-treatment with FBW for 24 hours and a post-treatment with QPt herbicide for another 24 hours.

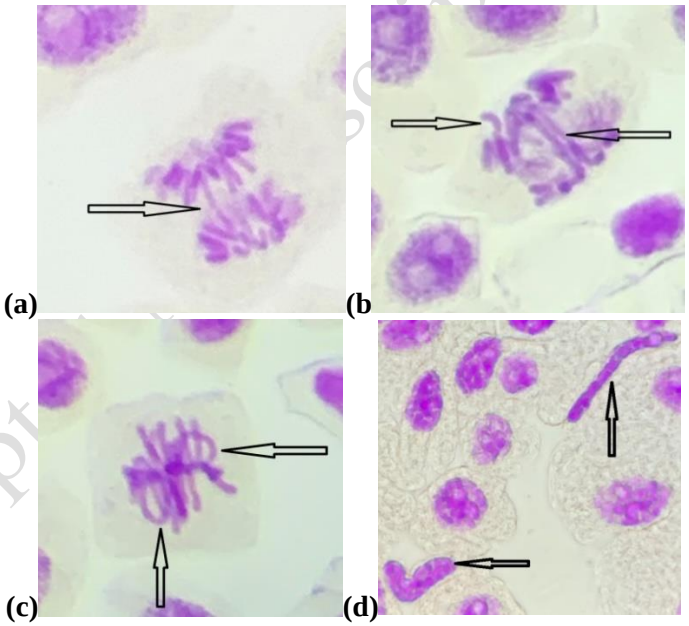

Figure 2. The main chromosomal aberrations and nuclear anomalies induced by pre-treatment with FBW and post-treatment with QPt in *A. cepa* cells: bridge (a), double bridge and laggard chromosome (b), ring chromosomes in metaphase (c) and strap nuclei (d). Arrows indicate abnormalities.