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TISSUE AND BIOCHEMICAL BARRIERS OF SUGAR BEET (*BETA VULGARIS* L. provar. altissima Doell.) PERICARP

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ABSTRACT

Shown that various pericarp of sugar beet hybrids contain a substantial amount of phenolic compounds which are represented by hydroxycinnamic acids, flavonoids and coumarins. Contained in the fruits of sugar beet triterpenoid saponins enhance the action of phenolic substances, creating complex system of protection of beet sprouts from the negative impact of biotic factors. The model of the functioning of biochemical regulation of seed germination and the formation around the pericarp phytoactive areas, performing the function of the protection system of seedlings from potential competitors and pathogens. A complex reaction of biological activity for water extracts of pericarp in relation to dicotyledonous plants, algae and root rots was established in the experimental data. This test can be used in the selection of sugar beet as a marker of potential resistance of genotypes to fungal and bacterial infections at the stage of seed germination.

Keywords: sugar beet, pericarp, phenolic compounds, biological activity, inhibitors, dormancy

INTRODUCTION

Seed viability saving of flowering plants during germination provided defence mechanisms (**Adkins *et al.*, 2002; Coumans *et al.*, 1976**), the most important of which are based on peculiarities of the structure of covering and mechanical tissues of the seeds, fruits and stems. (**Takhtajan, 1991**). One of the universal mechanisms that activate defence responses in plants to various fungal, bacterial, viral infections and insects as well as dormancy state control of seeds are thick endocarp cells, tannin and crystal-cells (**Barnabas *et al.*, 1990**). Electron microscopic studies have revealed close relationship of crystal-cells and sclereids in the process of histogenesis (**Klyachenko *et al.*, 2013**), conjugated to lignification of cell walls (**Gorshkova, 2007**).

Biologically active substances play an important role in the regulation of seed germination, growth and development of seedlings, hormones same how abscisic acid, ethylene (**Adkins *et al.*, 2002, Hermann *et al.*, 2007**), polyphenols (hydroxycinnamic acids, flavonoids, tannins, flavolignans) (**Blazhey *et al.*, 1977, Chiji *et al.*, 1980; Buer *et al.*, 2010**) coumarin (**Adkins *et al.*, 2002**) and saponins (**Vasilyeva *et al.*, 2000; Abid Ali Khan *et al.*, 2012**) are especially important. It was shown that highly active compounds can significantly affect germination of sugar beet seeds (*Beta vulgaris* L. provar. altissima Doell.), located mainly in the pericarp (**Chiji *et al.*, 1980**), and especially in the mesocarp tissues (**Inoue *et al.*, 1975**). Pericarps of sugar beet fruits are formed from the ovary walls, which are in the process of embryo formation, differentiate with formation of different tissue structures: exo-, meso- and endocarp (**Takhtajan, 1991**), functions of which are due to peculiarities of the anatomical structure, chemical nature and distribution of metabolites.

The most of seed germination inhibitors are polar compounds, which include abscisic (ABA) (**Coumans *et al.*, 1976**) and oxalic acids (**Hermann *et al.*, 2007**), oxalates, sodium nitrate (**Juntilla, 1976**) and phenol carbonic acids (**Morris *et al.*, 1984**). In the fruit of sugar beet there were detected hydroxycinnamic and hydroxybenzoic acids, protocatechuic and lilac aldehydes (**Chiji *et al.*, 1980**), cis-4-cyclohexane-1,2-dicarboximide (**Bewley D. *et al.*, 1980**) and 1-amino-1-cyclopropane carboxylic acid (key water soluble agent for maintaining of the dormancy state) (**Coumans *et al.*, 1976**). The most active inhibitors, according to some authors, are abscisic acid and coumarin which contained in fruit pericarps (**Kovalev *et al.*, 2003**). It was shown that the chemical compound from sugar beet pericarps can slow germination of seeds in many plant species (**Juntilla, 1976**). To the semi-polar inhibitors of beets flavonoids, bisalkaloids and isoindole compounds are belong.

Despite the fact that the main potential inhibitors of germination of sugar beet seeds are well known (**Barnabas *et al.*, 1990, Morris *et al.*, 1984**), concerning the mechanisms of seed germination regulation of sugar beet, insufficient and extremely contradictory information in the literature are presented. In this paper the results of research in the regulatory role of pericarp in seeds germination and formation of biochemical barriers of pericarp in different genotypes of sugar beet are represented.

MATERIAL AND METHODS

Two varieties of sugar beet Belotserkovskaya single seed 45 (BTS 45), Yaltushkovskaya single seed 64 (YlS 64) and 8 di- and triploid sterile hybrids on the basis of domestic and foreign selection: Ukrainskaya MS 70 (UkMS 70) and Ukrainskaya MS 72 (UkMS 72), Belotserkovskaya MS 57 (BTSMS 57), Ivanovskaya MS 33 (IvMS 33), Yaltushkovskaya MS 72 (YlMS 72), Uladovo-Verhnyachskaya MS 37 (UVMS 37) and Uladovo-Veselopodolianskaya MS 84 (UVMS 84), Alexandria.

The polar compounds from pericarps were extracted with bidistilled water at 40°C during 24 hours (in the ratio 1:10). The biological activity of the extracts was evaluated on the test object, using for this purpose pure cultures of Chlorella vulgaris 106 and Erwinia aroideae. Clean test cultures were plated on solid agar culture media. Aqueous extracts of fruits from examined genotype were added in amount of 30 mcl in prearranged holes 6 mm in diameter, and then test cultures were grown in incubator at a regulated temperature + 25°C. The biological activity of the extracted substance was evaluated according to the suppressed zone in the test culture on 5th, 7th and 12th day. Allelopathic potential (phytotoxicity) of water extracts was determined by the strength of the biologically active compounds influence on growth of radish sprouts' roots (Krasniy variety with white tip) (**Grodzinsky, 1973**).

Secondary metabolite profiling was performed by DAD-RP-HPLC on Agilent 1100 system using 2-eluent scheme (eluent A = 0,05 M aqueous solution of H3PO4; B = acetonitrile), column – Agilent Poroshell® 120, 2.7 µm, 2.1 × 150 mm, temperature control 20°C, sample volume 5 µl, flow rate 0.2 ml/min, analysis time up to 80 min, elution profile – wide range linear gradient from 0% B in A to 100% B in 30 min, then isocratic B with flow accelerated up to 0.6 ml/min and column temperature increased up to 40°C. Wavelength detection at 206, 254, 300, 350 and 450 nm was used to determine the most organic compounds (including terpenoids), most of substances with aromatic structure in molecule, phenylpropanoids (mainly cinnamic acids), flavonoids (flavones and flavonols),

carotenoids and chlorophylls, respectively. The spectra were recorded at peak maximum in the range 200-800 nm in order to elucidate the nature of secondary metabolites and attribute to certain groups of substances. It is not an exact chemical identification but the assumption based on the chromatographic behavior and spectra of separated components. Thus, flavones and flavonols were characterized by two distinct maximums at 260 and 350 nm. Lots of phenylpropanoids (hydroxycinnamic acids) and also isoflavones, flavanones, dihydroflavonols – by large maximum (often with shoulder) at 300-320 nm. Reproducibility of HPLC analysis was monitored using the mixture of nine alkylphenones (Sigma-Aldrich) from acetophenone to myristophenon. The error of sample injection did not exceed 2%, while the retention time deviation mostly ranged within 5%. Processing and visualization of chromatograms and absorption spectra were carried out using Agilent Chem Station and CorelDraw X3 software.

The chemical composition of the sugar beet fruits' metabolites was determined by TLC on the silica gel layer (Sorbfil F254) in the two types system of solvents: 1) chloroform – acetic acid – methanol – water (60:32:12:8); 2) ethyl acetate – acetic acid – formic acid – water (100:11:11:26) (**Kovalev *et al.*, 2003**). In order to identify the chemical nature of the substances chromatogram were treated with detection reagent (**Kovalev *et al.*, 2003**). Rf indicators (ratio of the covered distance by a substance from the starting line to the covered distance during the same time by a solvent system) were established according to photodensitometr using a computer program Sorbfil TLC. Presence of coumarins in methanol (MeOH) extracts was revealed with lactone sample and identification test on diazotised sulfanilic acid (**Kovalev *et al.*, 2003**).

The total content of phenolic compounds in fruits was determined spectrophotometrically using the Folin-Ciocalteu Reagent (Folin & Ciocalteu's phenol reagent) (**Singleton *et al.*, 1965**). The calibration diagram was built by gallic acid. The amount of flavonoids was determined in methanol extracts (1:10) at λ = 419 nm on the scanning spectrophotometer Optizen Pop successively adding 300 mcl of extract, 200 mcl of 0,1 M AlCl3 solution and 300 mcl of 1M CN3COONa. As a standard quercetin (Merk, Germany) was used. The dry solute substances content (Brix, %) in water extracts of fruits was determined with digital refractometer Reichert (USA).

Cytological analysis of pericarps and the state of test cultures was performed on a microscope Nikon Eclipse E-200. Autofluorescence of cells and tissues of pericarps were examined on microscope Axio Scope A-1 Carl Zeiss. Photo documentation of materials was carried out using a computer program Camera Control Pro-2 Nikon. The morphometric parameters of the lengths of the radish sprouts' roots (mm) and the results of bioassays were analyzed in specialized programs Image Pro-Premier 9.1 and AxioVision 4.7 Carl Zeiss. Statistical analysis was performed using Statistica 6.0 (trial version).

RESULTS AND DISCUSSION

Known secondary metabolites of sugar beet under researcher's interest are mainly presented by isoflavones, flavanones, dihydroflavonols and their glycosides (betavulgarin, betagarin, irisone B) with phytoalexin properties (antifungal antimycobacterial activity); betalain alkaloids (betalamic acid as betalain precursor, xanthins) with colourant and antioxidant properties; simple indole and bisindole alkaloids, isoindoles (Beta sp. germination inhibitor); di- and triterpenoids (saponins), for example glycosides of oleanolic acid, carophyllin, hederagenin and bisdesmosides (with antibacterial, ichthyotoxic and antiulcer properties) (**Dictionary of natural products, 2014**). In our study, in pericarps of sugar beet condensed tannins (proanthocyanidins), flavones, flavonols glycosides and glycosides of the latest were discovered (Figure 1).

Spectral characteristics of the aqueous extracts of fruits showed the absorption maxima (λ_{max}) in the range of 220 to 320 nm, indicative for hydroxycinnamic acids and coumarins, which was confirmed by quantitative and qualitative methods of analysis of phenolic compounds and flavonoids content which revealed significant genotypic differences (Table 1).

Table 1 Total content of dry soluble and phenolic substances in various seed pericarps of different sugar beet genotypes

Varieties, hybrids	Dry soluble substances, Brix, %	Phenolic substances, mg/g		Flavanoids, mg/g
		aqueous at +4ºC	methanolic	
Belotserkovskaya single seed 45	0.2 ± 0.02	22.8 ± 0,6	29.3 ± 0.7	4.9 ± 0.3
Yaltushkovskaya single seed 64	0.3 ± 0,02	21.2 ± 0,5	37.5 ± 0,7	11.5 ± 0.7
Ukrainskaya MS 70	1.3 ± 0.02	24.0 ± 0.7	31.6 ± 0.8	5.9 ± 0.4
Ivanovskaya MS 33	0.6 ± 0.03	32.0 ± 0.8	39.6 ± 0.9	3.2 ± 0.3
Yaltushkovskaya MS 72	0.3 ± 0.01	23.3 ± 0.4	28.0 ± 0.8	5.6 ± 0.5
Ukrainskaya MS 72	2.6 ± 0.02	22.5 ± 0.6	38.4 ± 1.2	5.9 ± 0.4
Belotserkovskaya MS 57	0.4 ± 0.02	21.8 ± 0.5	39.7 ± 1.0	9.7 ± 0.5
Uladovo-Verhnyachskaya MS 37	5.3 ± 0.10	29.3 ± 0.7	31.0 ± 0.8	14.9 ± 0.6
Alexandria	9.5 ± 0.12	22.9 ± 0.4	37.5 ± 1.1	12.5 ± 0.8
Uladovo-Veselopodolianskaya MS 84	4.5 ± 0.11	21.4 ± 0.7	38.3 ± 1.4	8.4 ± 0.5

Spectral characteristics of semi-polar compounds isolated from methanol extracts of the sugar beet pericarp are represented in Figure 1.

According to the general content of the dry saluted substances in pericarps there was a significant variation between beet genotypes which was explained primarily by the presence of various salts and organic acids in the pericarp composition. It was also found that the localization of secondary metabolites in the pericarps of studied hybrids of sugar beet was due to functional characteristics of tissues and cells. The outer layers of exocarp were composed from lignified cells with thickened walls, which were filled with intracellular polyphenolic compounds (Figure 2).

Mesocarp cells alive, palisade, with thin slightly lignified secondary cell walls containing plastids on the stage of the fruit ripening. There was also found numerous inclusions, crystals of calcium oxalate and druse. There were discovered a few hypha of the cell mycelium of endophytic fungi that moved from cell to cell through a pair of a simple unbranched pores. Signs of proteolytic tissue destruction of meso- and endocarp was not observed. In the endocarp cells which were represented by sklereids with numerous pore openings, also calcium oxalate crystals and vesicles filled with polyphenolic compounds were identified. From the periphery to the center sclereids reduced size, and cell membranes became more pigmented, thickened with a clear stratification. Formation of the secondary cell walls occured with participation of ferulic and p-coumaric acids which formed crosslinks between cellulose fibrils. Hydroxycinnamic acids along with associated form were also in free condition and easily transferred to the mobile phase through absorbing water by integumentary tissues. Accumulated in pericarp tissues, partially in vesicles, cell walls and in the intercellular spaces, phenolics, saponins and oxalates formed biologically active environment which condition depends on the content of free water in fetal tissues. The results showed that after water extraction of soluble compounds from the fruit, the highest germination of the sugar beet studied genotypes of sugar beet were observed in hybrids UVMS 37 and IvMS 33, pericarps of which contained the least amount of its residual amount. However, aqueous extracts of fruits of these hybrids provided the greatest inhibitory effect on the growth of roots of the radish test crops (Figure 3).

According to the principle of the discovered biologically active compounds in fruits on growth processes of radish roots, studied varieties and hybrids of sugar beet can be divided into two groups.

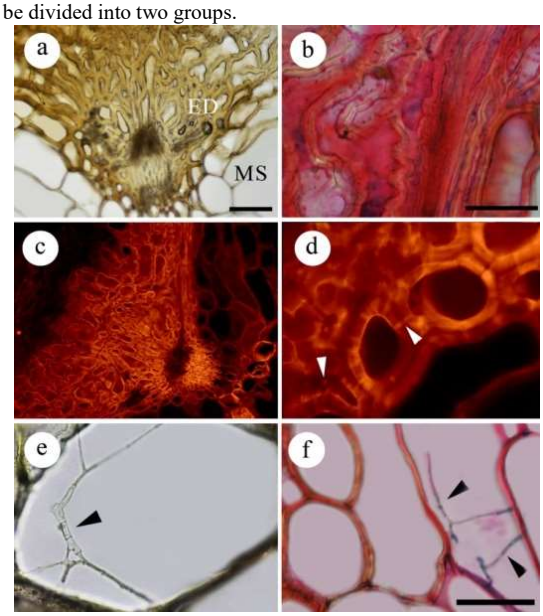

Figure 2 Peculiarities of the microscopic structure of meso- (MS) and endocarp (ED) (a-d) and the cellular mycelium endophytic fungi localization (e, f) in palisade cells of mesocarp in sugar beet fruits, where b and f - dyeing of tissues with safranin blue; c, f – fluorescence of sclereids, arrows show the intense glow of the space around the simple ostiole (emission spectrum - 640 nm)

The first was characterized by a general inhibition of root growth. It included varieties BTS 45, YlS 64 and hybrids UkMS 70, YlMS 72, Alexandria varieties. The second one had an inhibitory effect on a test culture with variable activity (Figure 3). It included IvMS 33, UkMS 72, BC 57 MS, UVMS 37, UVMS 84.

High negative correlation coefficient between the concentration gradient of fruit aqueous extracts and length of radish roots was noticed after 72 hours after seeds surviving (r = - 0,98). Between the total content of water-soluble phenolic compounds in fruit pericarp and length of radish roots correlation coefficient was r = - 0,72. Thus, there was not detected reliable relationship between the total content of dissolved dry substances including oxalates and phytotoxicity extracts (r = - 0,10). It was shown that out of 10 investigated genotypes of sugar beet in the pericarp of only five hybrids (IvMS 33, YalMS 72, UkMS 72, BTSMS 57, UVMS 37) and YlS 64 variety, contained complexes of compounds with distinct biologically active action (Figure 3).

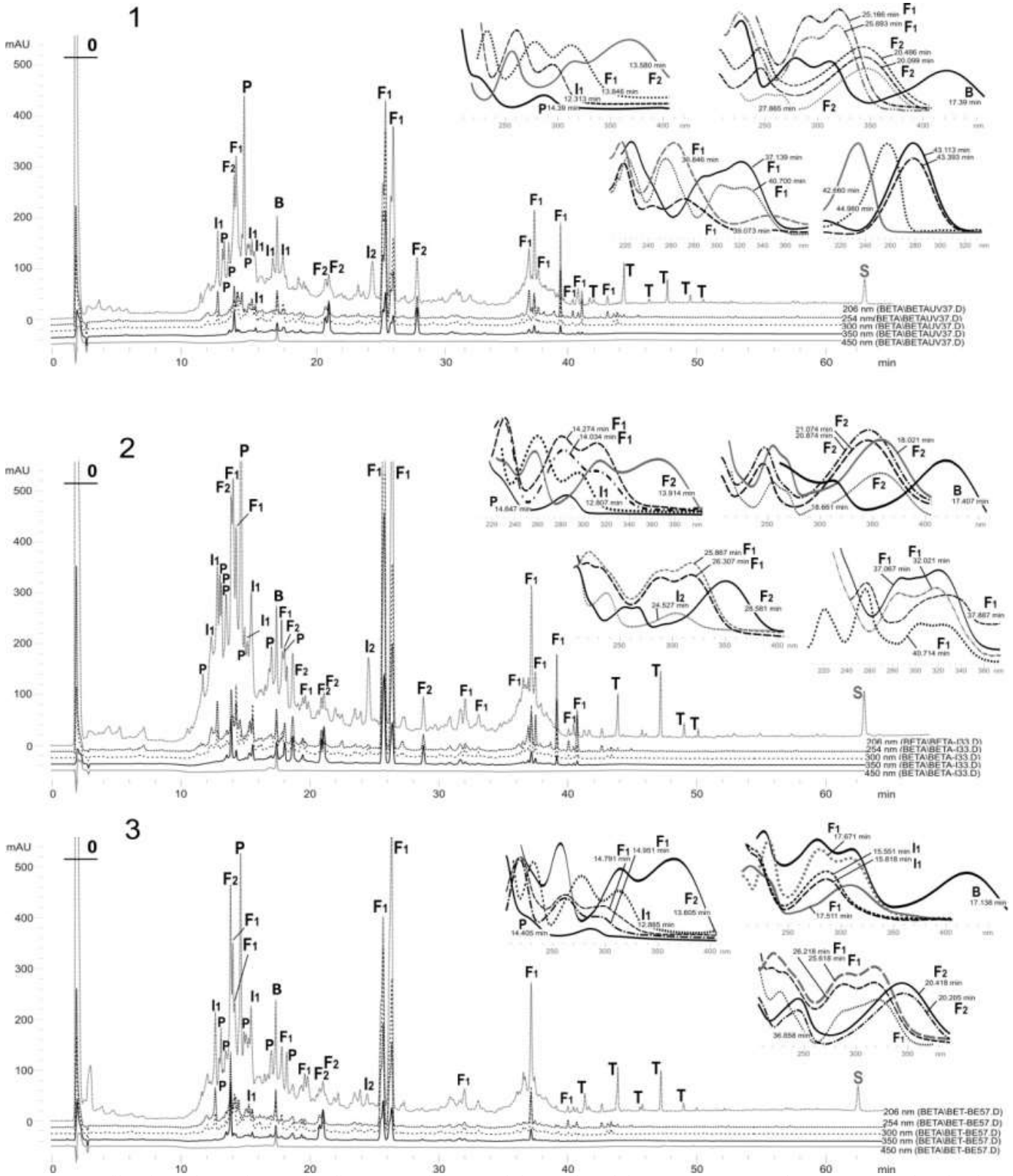

Figure 1 HPLC profiles of sugar beet pericarp methanolic extracts varieties: 1 – Uladovo-Verhnyachskaya MS 37; 2 – Ivanovskaya MS 33; 3 – Belotserkovskaya MS 57; component designations: 0 – components with no retention (mainly the pool of water-soluble acids etc.); B – betalamic acid; P – condensed tannins (proanthocyanidins); F1 – isoflavones, flavanones, dihydroflavonols and their glycosides (viz. irisone B, betavulgarin, betagarin); F2 – flavones, flavonols and their glycosides; I1 – simple indole and bisindole alkaloids; I2 – isoindoles (*Beta* germination inhibitor); T – di- and triterpenoids (saponins), viz. oleanolic acid and hederagenin glycosides and bisdesmosides; S – sterols and their esters. HPLC conditions are given in text. UV-VIS spectra of main components were shown. Assignment of the peaks has done according to the published data, chromatographic properties and spectral characteristics were credible but still requires further identification

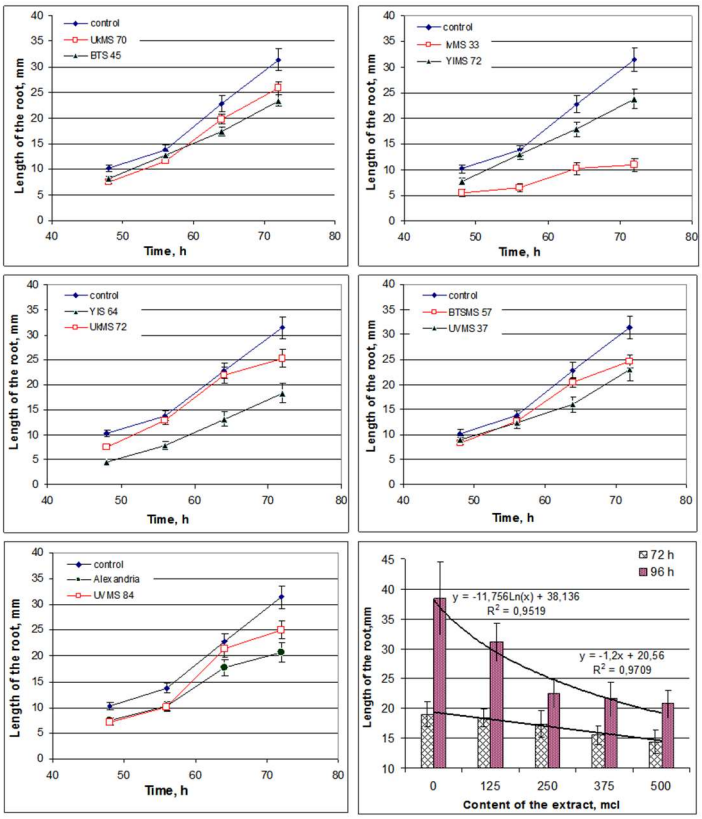

Figure 3 Inhibitory effect of aqueous extracts of the pericarp of different sugar beet genotypes on root growth of radish sprouts, where: a, e - extracts in solution 1:160; f - the root growth inhibition in the concentration gradient on the example of Uladovo-Verhnyachsky MS 37 hybrid extract

It's aqueous solutions in 100 times dilution retained the ability to foam abundantly both in acidic and in alkaline environment, which was indicative for triterpene saponins, aglycone of which in sugar beet is mainly oleanolic acid (**Mironenko *et al.*, 2011**).

Expressed phytotoxic and antibiotic action of metabolites of these genotypes was detected on pure test cultures *Chlorella vulgaris 106* and *Erwinia aroideae*. Test results have shown that the introduction of 30 mcl of water extracts in solid nutrient medium, in consequence of the free diffusion of metabolites, can inhibit its assimilation by the single-celled green algae for 4-5-week and bacterias up to 3 weeks. Microscopic analysis of culture *Chlorella vulgaris 106* showed that cells of the green algae in the zones of direct contact with endometabolites of the fruit pericarps of sugar beet slowly divided and formed small, uniformly dispersed aggregates, which included polymorphic cells.

Special attention must be given to the unique ability of pericarp aqueous extracts of hybrids IvMS 33, BTSMS 57, UVMS 37 of significant inhibition in the nutrient medium of *Erwinia aroideae* bacteria, representatives of which is the causative agent of the malicious disease of sugar beet root rot (**Klyachenko *et al.*, 2013**). Discovered complex represent biological activity of the fruit's pericarp aqueous extracts against dicotyledonous plants, algae and root rot which allow to use this test in selection of sugar beet as a marker of potential stability of genotypes to fungal and bacterial infections on the germination stage.

Analyzing dependence of the total phenolic content and area radius of algae cell division inhibition some patterns were determined based on the ratio of biologically active compounds in the pericarp of the sugar beet fruit and their effects on living organisms and the diffusion rate in the medium (Figure 4).

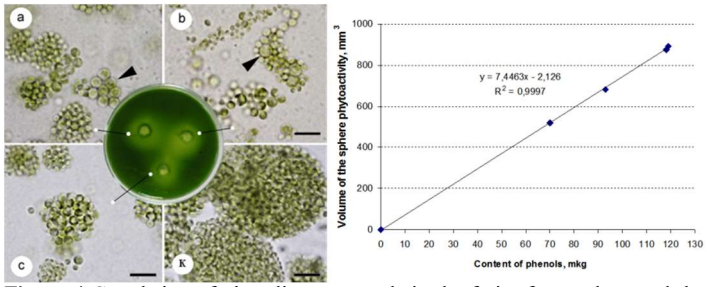

Figure 4 Correlation of phenolic compounds in the fruit of sugar beet and the radius of their phytotoxic action on *Chlorella vulgaris 106* in nutrient agar medium: a - IvMS 33; b - BTSMS 57; c - UVMS 37; k - control

Considering that the mass of the sugar beet fruit is an average from 11 to 25 mg, in which there are 900 mg of phenolic compounds and obvious phytotoxic and biocidal activities appeared at the concentration of substances – 100-150 g, the amount of potential phytoactivity sphere can be determined. Diffusion radius and phytotoxic action of biologically active compounds of sugar beet is function of its concentration in the fruits $R = f(C)$ and can be calculated for each genotype from indicators value of their total content in the pericarps. Volume of phytoactive sphere (V_{ph}) produced by fruits of studied hybrids can be determined by the following formula:

$$V_{ph} = 7,4463 \times C \times m - 2,126 ,$$

C – is concentration of phenolic compounds in fruits, m – fruit weight, 7.4455 and 1.905 – empirical parameters that determine the dependence of the zone of substances' active action on their concentration.

The total content of water-soluble phenols (hydroxycinnamic acids, tannins, flavonoids and coumarins), triterpene saponins and other biologically active compounds in the pericarp, is an important adaptive survival rate of seeds and seedlings of sugar beet. The phenolic complex and other active metabolites contained in pericarp tissues of beet fruits, under certain conditions, can function as a biochemical actuator (trigger) that at high concentrations supports dormancy of seeds, and low - stimulates germination. Based on the results of the research we have developed a model of formation of endogenous and exogenous biochemical barriers in pericarps of sugar beet fruits (Figure 5).

When water arrives in the pericarp tissue concentration of dissolved in them components will be quickly increased, which leads to increasing of intracellular osmotic potential of meso- and endocarp. In the case of insufficient water content only endogenous biochemically-osmotic barrier (bar_{en}) is created in the soil, which prevents the transport of water and oxygen to the tissues of the embryo and its germination initiation.

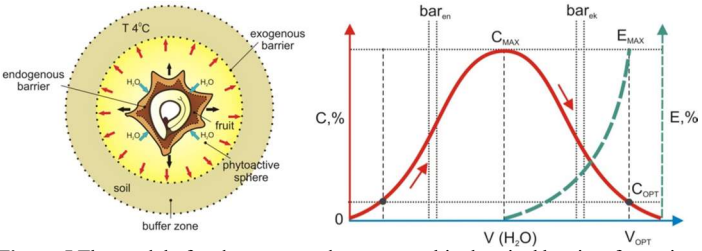

Figure 5 The model of endogenous and exogenous biochemical barriers formation which preventing development of pathogens inside and around the fruit of sugar beet as a result of the endometabolites diffusion: C – concentration in tissues of dissolved endometabolites (%); V (H_2O) – water volume in the fruit tissue; E – activation energy of seed germination (%); bar_{en} – endogenous (tissue) barrier; bar_{ek} – exogenous (phytogenous) barrier

At the same time dissolved phenolic and other active compounds inhibit the growth of endophytic fungi mycelium inhabiting cells of pericarp in the period of ripening and storage of fruit. At the optimum water content the outflow of water soluble organic compounds is accelerated and salts in the space around the fruit (phytogenic sphere), where the biologically active compounds create exogenous biochemical barrier (bar_{ek}), which controls reproduction of pathogenic microorganisms. This reduction in the concentration of phenolic compounds in fruit tissues, in contrast, stimulates growth processes, which favorably affects the viability of developing seedlings. In the context of insufficient water supply in the fruit of sugar beet the only one endogenous tissue biochemical barrier is formed, while ensuring optimal seed water supplementation initially formed endogenous and then exogenous (phytogenous) barrier.

CONCLUSION

Thus, as a result of the inducted research it was established that the majority of the studied genotypes of sugar beet in the fruit pericarps contained a complex of compounds with distinct biologically active action, most of which were salts of carboxylic acids and phenolic compounds: condensed tannins (proanthocyanidins), isoflavones, flavanones, digidroflavonoly, flavones, flavonoids and their glycosides and simple indoles and isoindoles (germination inhibitors), bis-indole alkaloids, di- and triterpenoids (hederagenin and oleanolic acid glycosides and bis-desmosides).

The model for formation of endo- and exogenous biochemical barriers which prevents pathogens development inside and around of sugar beet fruits during diffusion of endometabolites has been established. Adaptive parameters of seed germination, growth and development of sugar beet seedlings are the total content of water-soluble phenols, triterpene saponins and other biologically active compounds in pericarp which are capable to perform trigger functions under different conditions. High concentrations maintains the state of tranquility of seeds and low concentrations stimulates seed germination. Biological active compounds form endogenous (tissue) and exogenous (phytogenic spheres) biochemical

barriers by accumulating in tissues of meso- and endocarp of sugar beets. Ecological significance is active regulation of seed germination processes and suppression of phytopathogenic microorganisms on a formation stage of sugar beet seedlings.

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