The influence of plant protection by effective microorganisms on the content of bioactive phytochemicals in apples

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Abstract

BACKGROUND: The phytochemicals of two apple cultivars (Yellow Transparent and Early Geneva) protected in two ways, conventionally with chemical pesticides or by effective microorganisms (EM), were compared. Two types of components were determined: lipids synthesised constitutively and generated via inducible pathways polyphenols along with antioxidant activity and profiles.

RESULTS: The antioxidant activities assessed with ABTS, DPPH and Folin–Ciocalteu reagents were about two-fold higher in the case of microbiologically protected apples. The qualitative composition of phenolics determined by LC-DAD-MS varied between cultivars and the part of apples studied, while the method of protection caused mainly differences in concentration of some groups of polyphenols (hydroxycinnamates, flavanols, dihydrochalcones, flavonols, anthocyanins). The apples from biological cultivation contained about 34–54% more phenolics than these from a conventional orchard. In contrast, lipid composition did not differ significantly between apples originating from conventional and bio-crops.

CONCLUSION: The results indicate that the advantage of using the EM technology in agriculture may not only be the reduction of consumption of chemical fertilisers and synthetic pesticides, but also, at least in the case of apples, may lead to the production of crops with improved health quality due to the higher content of bioactive phytochemicals.

Keywords: effective microorganisms; apples; antioxidants; polyphenols; lipids

INTRODUCTION

Pesticides, as well as nitrates (III) and (V) are listed among the main groups of environmental pollutants found in food and drinking water. These important agri-economy compounds are known to pose a health risk to animals and humans. In particular, such an exposure is suggested to increase cancer incidence both in people from rural areas professionally exposed to pesticides, as well as in consumers who ingest pesticides and nitrates present in food products.[1] Therefore, the search for more environmentally friendly sustainable alternatives to the current agricultural practices is an active field of research, in addition to regulatory activities. For instance, the regulations recommended as a result of the provisions of Art. 14 of Directive 2009/128/EC and Regulation No 1107/2009 to apply the principles of integrated pest management have been introduced in the majority of European countries. In agriculture, the principles of integrated production aim at the reduction of consumption of chemical pesticides and fertilisers. However, to maintain reasonable economical profitability, alternative methods of soil enrichment and crop protection must be implemented. One of the proposed approaches is organic fertilisation or bio-based agents exploiting anti-biological properties of microorganisms. The bio-based technology which appears to fulfill requirements faced by a novel complete sustainable production system, i.e. ensuring proper soil quality and crop protection, may be supported with an appropriate complex of beneficial microorganisms. Such a mixed inoculum, known as 'effective microorganisms', has been on the market in a number of countries for some time and its aim is to improve plant health and soil productivity without harming the environment, being safe for human consumption at the same time.

Pioneering work in advancing the concept of 'effective microorganisms' (EM) was conducted by Teruo Higa, Professor of Horticulture, University of the Ryukyus, Okinawa, Japan. He developed the microbial inoculants and demonstrated their ability to improve soil quality, crop growth, and crop yield.[2,3] His work has quickly gained the attention of environmentalists worldwide. EM are a fermented mixed culture composed of non-pathogenic microbial species capable of coexisting in acidic medium (pH

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below 3.5). Among the main microorganisms constituting an EM culture, there are the species of photosynthetic bacteria (*Rhodopseudomonas plastris* and *Rhodobacter sphacrodes*), lactobacilli *(Lactobacillus plantarum*, *L. casei* and *Streptococcus lactis*), yeasts (*Saccharomyces* spp.), and actinomycetes (*Streptomyces* spp.). The exact composition of the EM preparation is not disclosed; however, a growing number of companies offer EM-based preparations, hence it can be presumed that their production must be strictly controlled. Nonetheless, the literature describing the results of purposefully designed investigations verifying environmental and nutritional benefits of EM technology is very limited. Some studies demonstrated the positive influence of EM on agricultural production and ascribed it to the improvement of physical, chemical and biological environments of the soil and suppression of soil-borne pathogens and pests.[4,5] The enhancement of germination of seeds, flowering, fruiting and ripening in the case of plants treated with EM was also observed.[6,7] According to other authors, the application of EM strengthened the photosynthetic capacity of crops, increased crop yield and resistance to plant diseases.[8,9]

In spite of these promising advantages, EM-based products have not so far been effectively exploited at larger scales, neither to improve plant yields, nor to prevent degradation of areas of agricultural importance. Even less is known about the impact of microbial protection of crops on health quality of foods based on raw materials derived from cultivations treated in this way. The healthiness of plant crops is related not only to nutritional/toxic components ratio, but also to non-nutrient bioactive secondary metabolites whose biosynthesis may be affected by abiotic challenges, but is particularly responsive to biotic stress.[10] For instance, fungal infection or mock inoculation (*Colletotrichum acutatum* and *cinerea*) of strawberries seemed to influence the expression of phenylpropanoid, flavonoid and shikimate pathway genes.[11] Similarly, the recent studies by Ali and McNear[12] for *Arabidopsis* showed that this time purposeful additions of microbially based soil additives (species of bacillus, actimomyces and proteobacteria included in Soil Builder™-AF) have a perceptible influence on phenylpropanoid pathway gene regulation resulting in the enhancement of production of secondary metabolites. These results indicate that EM use in crop production could also enhance functional and health-promoting value of edible plants by stimulation of biosynthesis of bioactive phytochemicals, e.g. polyphenols.

Poland is the largest producer of apples in Europe. Therefore, here, it may be of major interest to agri-business whether the replacement of the current method of protection of orchards with EM-based products, representing one of the suggested sustainable technologies, may improve health value, and hence the attractiveness of these fruits to consumers. In our study, we compared phytomes of two apple varieties derived from two commercial orchards applying different methods of protection: conventional with synthetic pesticides or alternative with EM preparation (EmPharma Plus™). The health quality was assessed based on antioxidant activity, as well as content and composition of polyphenols as measures reflecting the chemopreventive potential of fruits. However, biosynthesis of these phytochemicals relies on inducible pathways, and hence may occur to very variable extents even in individual apple fruits from the same tree. Therefore, as a sort of internal control, in seeds and peels, we also characterised composition of lipid fraction whose synthesis is expected to be constitutive.

MATERIALS AND METHODS

Reagents

HPLC grade methanol, chloroform, 2-propanol, *n*-hexane and formic acid (98–100%) were obtained from Merck (Darmstadt, Germany). Potassium chloride, anhydrous sodium sulfate, diethyl ether, acetic acid were purchased from POCh (Gliwice, Poland) and borontrifluoride–methanol solution (14% in methanol) from Sigma–Aldrich (St Louis, MO, USA) were of analytical grade. The following standards were used: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), L-ascorbic acid, (–)-epicatechin, (+)-catechin, quercetin-3-*O*-galactoside, phloridzin, triacylglycerol (TAG, triolein), diacylglycerol (DAG, 1,2-diolein), monoacylglycerol (MAG, 1-monoolein), sterol esters (SE), phytosterols (P, *β*-sitosterol), free fatty acid (FFA, oleic acid), wax ester (WE, oleyl linoleate) and phospholipids (PL, phosphatidylcholine) from Sigma–Aldrich (USA); cyanidin-3-*O*-glucoside, procyanidin B1, procyanidin B2, *p*-coumaric acid from Fluka (Saint Louis, MO, USA); chlorogenic acid from Extrasynthese (Lyon, France); FAME standard solutions (Supelco 37 Component FAME Mix, FAME Mix C20:1 to C20:5, PUFA No.1 Marine Sources) from Supelco (Bellefonte, PA, USA) and FAME standard solutions (Mixture ME 62 Mixture ME 64 Mixture ME 81) from Larodan Fine Chemicals AB (Malmö, Sweden). The reagents used for the detection of antioxidants included 2,2′-azinobis(ethyl-2,3-dihydrobenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), both from Sigma–Aldrich, and Folin–Ciocalteu phenol reagent (FCR) from Merck. The DPPH radical stock solution was prepared in methanol (5 mmol L^{-1}) just before the experiments and kept in a lightproof container. The stock solution of ABTS was prepared in aqueous $Na_2S_2O_8$ solution (2.45 mmol L^{-1}) to reach a concentration of 7 mmol L^{-1} and left in the dark at ambient temperature. Under such conditions, the concentration of ABTS radical reaches a maximum after 6 h and is stable for >2 days. FCR commercial solution was diluted with water (1:9, v/v) just before use.

Plant material and sample preparation

Two cultivars of apple (Yellow Transparent and Geneva Early) were harvested at the stage of maturity in the two neighbouring commercial orchards (Grudziądz region, North-Western Poland); one protected by conventional pesticides (no details as regards time schedule or applied chemicals could be obtained) and the other since 2006 solely by EM preparation dedicated to plant protection (EmFarma Plus™; ProBiotics Poland, Pawłowo, Poland). In Poland, the safety of EmFarma Plus™ has been positively evaluated by the National Institute of Public Health (attest Nr PZH/HT-3112/2016). The microbiological protection in the latter orchard was performed for the eighth season and involved application of 10× diluted with the tap water commercial EmFarma Plus™ preparation as follows: three times before blossom time and twice during blossom. For the study, the pooled plant material, representing five randomly chosen apples from about a 15 kg portion, was used. Fresh fruits were peeled and divided into three fractions, peel, flesh and seeds. Peel and flesh samples were then freeze-dried, crushed and kept at −20 °C until analysis. For the determination of antioxidant activity and content of phenolics, lyophilised plant material (0.5 g) was extracted three times with methanol (6 mL). This solvent was chosen for extraction according to recommendations published earlier.[13] After each step of extraction, samples were centrifuged (1690 × *g*, 15 min) and clear supernatants collected. Apple seeds were kept at −20 °C until extraction of lipids.

Determination of antioxidant activity

The colorimetric determination of antioxidant activity was performed by the standard methods employing ABTS, DPPH, and FCR indicators as described earlier.[14] The antioxidant activity of apple samples was calculated based on standard lines generated for Trolox and expressed as Trolox equivalents (TE, μmol g^{-1} DW).

Determination of phenolic compounds and vitamin C by high-performance liquid chromatography

The HPLC system applied for phenols and ascorbic acid determination consisted of an Agilent 1200 series photodiode array detector and mass spectrometer (Agilent 6130 Quadrupole LC/MS) equipped with an electrospray ionisation interface. An Agilent Eclipse XDB-C18 column (4.6 mm × 150 mm) with 5 μm particle size and 10 μL injection volumes were used. The mobile phase contained aqueous 48 mL L^{-1} formic acid (A) and methanol (B). The gradient applied was 5–50% B in 20 min and 50–100% in 5 min at a flow rate of 0.8 mL min^{-1}. Absorbance spectra were recorded between 190 and 700 nm every 2 s with a bandwidth of 4 nm, while the chromatograms were monitored at 254 nm for ascorbic acid, at 280 nm for catechins, procyanidins and dihydrochalcons, at 325 nm for hydroxycinnamic acid derivatives, at 360 nm for flavonols and at 525 nm for anthocyanins. MS parameters were as follows: capillary voltage, 3000 V; fragmentor, 120 V; drying gas temperature, 350 °C; gas flow (N_2), 12 L min^{-1}; nebuliser pressure, 35 psig. The instrument was operated in positive ion mode, scanning from *m/z* 100 to 1000. The peaks were identified by comparison of retention times and UV spectra with those of authentic reference substances or on the basis of available literature data and mass spectra. The quantification of the analytes for which standards were available was performed with external calibration curves generated by integration of the areas of absorption peaks, whereas for analytes for which standards were lacking by reporting the measured chromatographic area in the calibration equation of the reference standards (phloretin-2-*O*-xyloglucoside was quantified as phloridzin at 280 nm, 4-*p*-coumaroyloquinic acid was quantified as *p*-coumaric acid at 325 nm; quercetin derivatives were quantified as quercetin-3-*O*-galactoside at 360 nm and unknown procyanidins were quantified as (+)-catechin at 280 nm).

Profiling of antioxidants by postcolumn derivatisation

The profiling of antioxidants in apple extracts was performed by post-column derivatisation as described earlier[14–16] with slight modifications. The post-column addition of ABTS derivatisation reagent to HPLC eluate was performed using a Pinnacle PCX derivatisation instrument (Pickering Laboratories Inc., Mountain View, CA, USA). The derivatisation reagent was prepared by dilution of ABTS stock solution (7 mmol L^{-1}) with methanol to a concentration of 300 mL L^{-1}. Derivatisation was carried out at 130 °C with the flow rate of derivatisation reagents set at 0.2 mL min^{-1}. The 0.5 mL (PTFE, 0.25 mm, 10 m) coil available as a standard part of the Pinnacle PCX derivatisation instrument was used. Chromatograms of the products formed after derivatisation of antioxidant compounds with ABTS reagent were registered at 734 nm using a multiple-wavelength detector (Agilent 1200 series MWD, USA).

Total lipid extraction

The total lipids were isolated from the freeze-dried peel samples or seeds by Bligh and Dyer's method[17] modified by Christie[18] with the aid of chloroform/methanol extraction mixture and potassium chloride solution. To 1 g portions of crushed samples (seeds or peel), 5 mL of chloroform, 10 mL of methanol and 5 mL of 8.8 g L^{-1} potassium chloride water solution were added. The suspension was stirred in a shaker for 3 min. Then, 5 mL of chloroform and 4 mL of 8.8 g L^{-1} sodium chloride water solution were added. Once again, the mixture was stirred in a shaker for 2 min and transferred into a separating funnel. After separation of the phases, the bottom phase was filtered into a flat-bottom flask through filter paper containing anhydrous sodium sulfate. The solvent was removed in a rotary evaporator at 40 °C. The oil was placed in a glass vial filled with nitrogen and stored at −18 °C until analyses.

Determination of fatty acid composition

The composition of fatty acids was determined by gas chromatography. Fatty acids were separated as fatty acid methyl esters (FAMEs) prepared according to the standard procedure (EN: ISO, 5509 2000).[19] The analysis was conducted using a Hewlett-Packard 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a split/splitless injector, Rtx 2330 column (100 m × 0.25 mm; Restek, Bellefonte, PA, USA) and flame ionisation detector (FID). The FID and injector temperature were both maintained at 250 °C. The initial column oven temperature was 180 °C for 20 min, then gradually was increased to 210 °C at 1.5 °C min^{-1} rate and held at this temperature until the analysis was completed. A qualitative and quantitative determination of fatty acid content was performed based on the analysis of standard FAME solutions. The content of fatty acids was expressed as % of the total FAMEs.

Extraction of waxes

The waxes contained in apple seeds and peels were separated from the total lipids using SPE method described by Reiter *et al.*[20] Approximately, 30 mg of the total lipids was dissolved in 0.5 mL of *n*-hexane. The solution was applied onto silica cartridges (500 mg, 3 mL, Backerbond; J.T. Baker, Deventer, Netherlands) preconditioned with 3 mL of *n*-hexane. The samples were passed through the column and the retained waxes were then eluted with 10 mL of *n*-hexane/diethyl ether (99:1, v/v). The solvent was evaporated in a stream of nitrogen and the content of wax esters was determined on the basis of the weight of residues. After drying of the SPE column, the remaining lipid classes were eluted with 15 mL of chloroform/methanol (2:1, v/v). The solvents were evaporated in a stream of nitrogen. The lipids were weighed and dissolved to obtain concentration of 10 mg mL^{-1} in chloroform/methanol (2:1, v/v). This solution was used for HPLC analysis of lipid classes. The purity of eluted fractions was confirmed by TLC (silica gel TLC 60 F254, 0.25 mm; Merck) using a hexane/diethyl ether/acetic acid (80:20:2, v/v/v) as a mobile phase.[21]

HPLC analysis of lipids

Lipid separation was accomplished by a reversed-phase HPLC method described by Perona and Ruiz-Gutierrez.[22] A Perkin-Elmer chromatograph (Perkin-Elmer, Waltham, MA, USA) equipped with LiChrosphere 100 Diol (250 × 4.6 mm, 5 μm particle size; Merck) and controlled by the TCNav system was used. A 10 μL of sample was injected onto the column using an appropriate sample loop. A laser light-scattering detector LLSD (BTT Automatyka, Gdansk, Poland) was used for the detection. The detector temperature was set at 60 °C; carbon dioxide pressure was 140 kPa. The column temperature was maintained constant at 30 °C and the flow rate

was 0.8 mL min^{-1} during all runs. For the separation of lipids, three solvents in the gradient elution were used: (A) *n*-hexane, (B) isopropanol, (C) methanol. The gradient applied was as follows: 99.2% A, 0.8% B in 0–3 min; 97% A, 3% B in 3–8 min; 90% A, 10% B in 8–13 min; 70% A, 30% B in 13–30 min; 60% B, 40% C in 30–45 min. Calibration curves were generated for lipid standards including: TAG, DAG, MAG, SE, P and FFA prepared in chloroform/methanol (2:1, v/v) in the concentration range of 0.025–5.0 mg mL^{-1}. The contents of lipid classes were expressed as mass % of total lipids.

Statistical analysis

All determinations were carried out in three parallel replications and mean ± SD were calculated for the values obtained. By using one-way ANOVA, the significance of differences was calculated between mean values of results obtained during determinations for apple tissues originating from conventionally and microbiologically protected orchards. Analysis of variance was performed with Tukey's multiple comparison test. The GraphPad Prism 6 was used for data analysis (La Jolla, CA, USA).

RESULTS AND DISCUSSION

The investigations were designed to compare the composition of the phytomes of two popular cultivars, the earliest ripening cultivars of Polish apples originating from orchards protected either chemically or microbiologically. These were a pale yellow cultivar Yellow Transparent and a dark red cultivar Geneva Early collected from two neighbouring orchards in Northern Poland employing different methods of protection; either with chemical pesticides (Conv.) or EM preparation (EM). Although in many studies in which cultivars have been compared, polyphenols were extracted from a fruit as a whole, we decided to analyse different parts of fruits separately. Peel and flesh each provide a significant contribution to the total amount of consumed polyphenols; however, phytochemical composition in each tissue can be quite different.[23,24] Accumulation of soluble phenolics is greater in the outer tissues (epidermal and sub-epidermal layers) than in the inner tissues (mesocarp and pulp) and it may be additionally enhanced by microbial inoculum applied superficially in the case of EM-protected apples, because biosynthesis of secondary metabolites is inducible in response to environmental challenges. The lipid fraction, in particular in seeds, is generated rather constitutively, so could be expected to be less responsive to the method of orchard protection. This suggests that accumulation of these groups of compounds in each tissue may be under different genetic control, and the responsiveness of their accumulation to environmental factors may also be tissue dependent.[25] In this study, we determined the antioxidant activity, phenolic composition and chromatographic profiles of antioxidants in peel and flesh tissue, as parameters strongly affected by environmental conditions while the content and composition of lipids in peel and seeds as a sort of internal control that mirrors natural variability of plant material.

Phenolics and vitamin C content

The comparison of vitamin C and polyphenol composition and content in the extracts from flesh and peel of two apple cultivars originating from orchards conventionally or EM-protected are presented in Table 1.

The method of orchard protection had no influence on the chemical composition of phenolic compounds in both varieties of apples studied. The HPLC-DAD analysis revealed the presence of flavanols, flavonols, dihydrochalcones, and hydroxycinnamic acids, as well as anthocyanins in the red cultivar. The peaks exhibiting high UV-absorbing properties were putatively identified by LC-MS based on their mass spectra and available literature data.[26,27] Mass spectra of the polyphenols detected in the apple extracts provided data about molecular weights and constitutive units of analytes detected. APCI mass spectra of polyphenols in the positive ion mode under the experimental conditions usually showed protonated molecular ions as a main peak $[M + H]^+$ (Table 1). These assignments were then confirmed by comparing HPLC-DAD retention times and UV spectra with those of authentic standards when available.

During analyses several well-resolved chromatographic peaks of flavan-3-ols were detected (Fig. 1). In the mass spectra of catechins (flavan-3-ol monomers), the main protonated molecular ion at *m/z* 291 indicated the presence of (+)-catechin and (–)-epicatechin (peaks 4 and 10, respectively). The other flavan-3-ols detected in extracts were dimers, at *m/z* 579 (peaks 2, 5, 6, 9, 13), and trimers, at *m/z* 867 (peaks 3 and 8). Among procyanidins, only procyanidin B1 and B2 were identified by comparison with their standards (peaks 2 and 6, respectively). The other group of flavonoids present in apple extracts were dihydrochalcones, two of which were identified as phloretin-2-*O*-xyloglucoside (peak 14) and phloretin-2-*O*-glucoside (phloridzin, peak 17). Their mass spectra revealed protonated molecular ions at *m/z* 569 and 437, respectively. Among flavonols, the identified quercetin glycosides included quercetin-3-*O*-galactoside (hyperoside, peak 15, *m/z* 465), quercetin-3-*O*-xyloside (peak 16, *m/z* 435), quercetin-3-*O*-arabinoside (peak 18, *m/z* 435) and 3-*O*-rhamnoglucoside (peak 18, *m/z* 449). Two hydroxycinnamic acids were also detected in apple samples studied, 5-caffeoylquinic acid (peak 7, *m/z* 355) and 4-*p*-coumaroylquinic acid (peak 11, *m/z* 339). In the red cultivar Geneva Early, anthocyanin cyanidin-3-*O*-galactoside (peak 12, *m/z* 449) also was identified, both in peel and flesh samples.

The content of phenolic compounds varied greatly among the corresponding apple samples studied, both in peel and flesh. In the cases when these values differed by at least 100%, they are indicated by the grey background in the Table 1. The total phenolic content in apples from conventional orchards ranged from 17.55 to 21.93 mg g^{-1} DW (1.84–2.64 mg g^{-1} FW) in the flesh and from 19.30 to 31.75 mg g^{-1} DW (3.42–7.24 mg g^{-1} FW) in the peel, for yellow vs. red cultivars, respectively. Such amounts are in accord with literature data that also indicated wide range of determined levels of polyphenols. For instance, the apples derived from Italian areas were shown to contain between 0.66 and 2.12 mg g^{-1} FW of such secondary metabolites according to Vrhovsek *et al.*[26] or 0.51–1.06 mg g^{-1} FW in flesh and 2.32–5.76 mg g^{-1} FW in peel according to Lamperi *et al.*[28] Tsao *et al.*[29] determined the total phenol level in eight apple cultivars in the range of 0.18–0.93 mg g^{-1} FW in flesh and 1.02–2.07 mg g^{-1} FW in peel. In Golden Delicious apple after pressurised liquid extraction (PLE), these values ranged between 1.34 mg g^{-1} DW for pulp and 7.57 mg g^{-1} DW for peel.[30] For whole apples, the reported total phenol levels depended on the country of origin, thus probably on variety, and amounted to 0.38–0.92 mg g^{-1} FW in the USA;[31] 1.31–2.50 mg g^{-1} DW in Germany,[32] or 5.23–27.24 mg g^{-1} DW in Poland.[33]

In the case of both apple cultivars derived from orchards protected by EM, the phenolic contents were similar in flesh of both cultivars: 33.28 to 34.17 mg g^{-1} DW (4.23–4.35 mg g^{-1} FW), but differed substantially and were much higher in the peel of red variety: 40.72 vs. 64.67 mg g^{-1} DW (8.65 vs. 16.76 mg g^{-1} FW) (Table 1).

Table 1. Composition and content (mg g^{-1} DW) of phenolic compounds and vitamin C detected in flesh and peel samples of two cultivars of apple derived from orchards protected by conventional pesticides (Conv) or by effective microorganisms (EM) compiled with chromatographic and spectrometric data

				Yellow Transparent						Geneva Early					
				Flesh			Peel			Flesh			Peel		
Peak no	Rt (min)	Compound	$[M+H]^+$	Conv	EM	*P*	Conv	EM	*P*	Conv	EM	*P*	Conv	EM	*P*
1	1.9	Vitamin C	177	3.98	2.94	****	2.27	2.13	NS	2.92	2.65	**	2.32	2.27	NS
2	6.8	Procyanidin B1	579	0.52	0.49	NS	ND	ND	–	0.37	0.39	NS	ND	0.62	****
3	8.0	Procyanidin trimer	867	0.79	0.51	*	1.04	0.86	NS	0.82	0.61	*	0.98	0.93	NS
4	8.3	(+)-Catechin	291	0.57	0.55	NS	0.73	0.87	NS	0.73	0.79	NS	0.80	1.60	–
5	8.6	Procyanidin dimer	579	0.37	0.71	**	0.76	1.01	**	0.50	0.87	****	0.84	1.45	****
6	9.3	Procyanidin B2	579	0.79	1.96	****	1.11	2.47	****	3.10	4.21	****	2.97	3.38	****
7	9.9	5-Caffeoylquinic acid	355	7.66	17.79	****	3.81	10.27	****	2.36	4.03	****	0.72	2.08	**
8	11.0	Procyanidin trimer	867	1.05	1.87	****	1.70	4.04	****	4.50	7.16	****	4.63	12.20	****
9	11.4	Procyanidin dimer	579	ND	ND	–	ND	ND		2.66	4.50	****	5.64	12.35	****
10	11.6	(–)-Epicatechin	291	2.25	2.38	NS	2.31	3.69	****	5.39	9.03	****	9.58	18.76	****
11	12.6	4-*p*-Coumaroyloquinic acid	339	2.20	5.75	****	0.94	4.40	****	0.37	0.31	NS	0.14	0.17	NS
12	13.0	Cyanidin-3-*O*-galactoside	449	ND	ND	–	ND	ND	–	0.04	0.23	NS	0.45	1.06	****
13	15.3	Procyanidin dimer	579	ND	ND	–	ND	ND	–	0.83	0.66	NS	0.83	0.61	NS
14	17.9	Phloretin-2-*O*-xyloglucoside	569	0.76	1.24	****	0.80	1.59	****	0.25	0.48	*	0.36	0.68	*
15	18.4	Quercetin-3-*O*-galactoside	465	ND	ND	–	1.47	1.53	NS	ND	ND	–	0.59	1.41	****
16	19.1	Quercetin-3-*O*-xyloside	435	ND	ND	–	0.63	1.13	****	ND	ND	–	0.28	0.77	***
17	19.3	Phloridzin	437	0.58	0.93	**	0.54	3.54	****	ND	ND	–	ND	ND	
18	20.1	Quercetin-3-*O*-arabinoside	435	ND	ND	–	2.56	4.17	****	ND	ND	–	1.70	3.76	****
19	20.5	Quercetin-3-*O*-rhamnoside	449	ND	ND	–	0.90	1.15	**	ND	ND	–	1.23	2.86	****
Total phenolics				17.55	34.17	****	19.30	40.72	****	21.93	33.28	****	31.75	64.67	****

P, significant differences between corresponding samples (Conv vs. EM); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$; NS, not significant; ND, not detected.
The differences in the content of compounds in corresponding samples (Conv vs. EM) higher than 100% are marked by grey background.
Standard deviation <1.0% for triplicate determinations.

Among the five major groups, the flavanols predominated in both peel and flesh of 'Geneva Early' and ranged from 80% to 86%. These data are in agreement with other authors.[28,33,34] In the case of 'Yellow Transparent', hydroxycinnamic acids together with chlorogenic acid were dominating constituents (from 25% to 36% in flesh and from 56% to 69% in peel). The highest content of hydroxycinnamic acids in phenolic fraction of apples was also reported by other authors.[35,36] The Yellow Transparent cultivar, had also higher than Geneva Early content of dihydrochalcons group, which represented 6.3–12.6% of total phenolics. The flavonols were determined only in peel samples of both studied cultivars. This group of phenolics was a mixture of four different quercetin glycosides with quercetin-3-*O*-arabinoside as predominant flavonol.

The levels of each group of phenolics were higher in both cultivars of apples protected by EM than in apples protected by pesticides. In the case of Yellow Transparent cultivar, the content of flavanols, hydroxycinnamic acids and dihydrochalcons were about 25, 58 and 38% higher in flesh and 41, 68 and 74% in peel samples, respectively. The levels of flavanols, hydroxycinnamic acids, dihydrochalcons and also anthocyanins in the case of 'Geneva Early' apples protected by EM were higher than in conventional apples by about 33, 37, 48 and 85% in flesh and 49, 62, 47 and 57% in peel samples, respectively. The flavonol contents were also higher in peel of both cultivars from EM orchards; by about 30 and 57% in Yellow Transparent and Geneva Early cultivars, respectively.

The identical composition of bioactive polyphenolic compounds in apple samples, regardless of the way of orchard protection, suggests that none of the biochemical pathways involved in biosynthesis of these phytochemicals was shut down. However, the comparison of quantities of phenolic components indicated that EM application must have stimulated at least some of enzymatic activities responsible for generation of secondary metabolites compared to treatment with conventional pesticides. The levels of total phenols were on average two-fold higher in EM-protected fruits than those determined for fruits from conventionally protected orchards. This stimulation was particularly visible in the peels, i.e. tissue in direct contact with applied microbial preparation. In contrast, the application of EM had little effect on vitamin C abundance, whose content appeared slightly smaller in apples from the microbiologically protected orchards and was in agreement with earlier reports where vitamin C content in different apple varieties ranged from 0.12 to 0.35 mg g^{-1} FW[37] or from 0.003 to 0.75 mg g^{-1} FW.[38] The determined values for this analyte ranged from 2.13 to 3.98 mg g^{-1} DW, which corresponds to 0.35–0.59 mg g^{-1} FW.

The important influence of the cultivation type on the level of polyphenols in apple fruits and leaves has been demonstrated previously. For instance, apples from organic production, thus more exposed to biotic stress, showed typically higher content of hydroxycinnamic acids, flavanols, dihydrochalcones, quercetins and total phenolics than apples from integrated cultivation.[39]

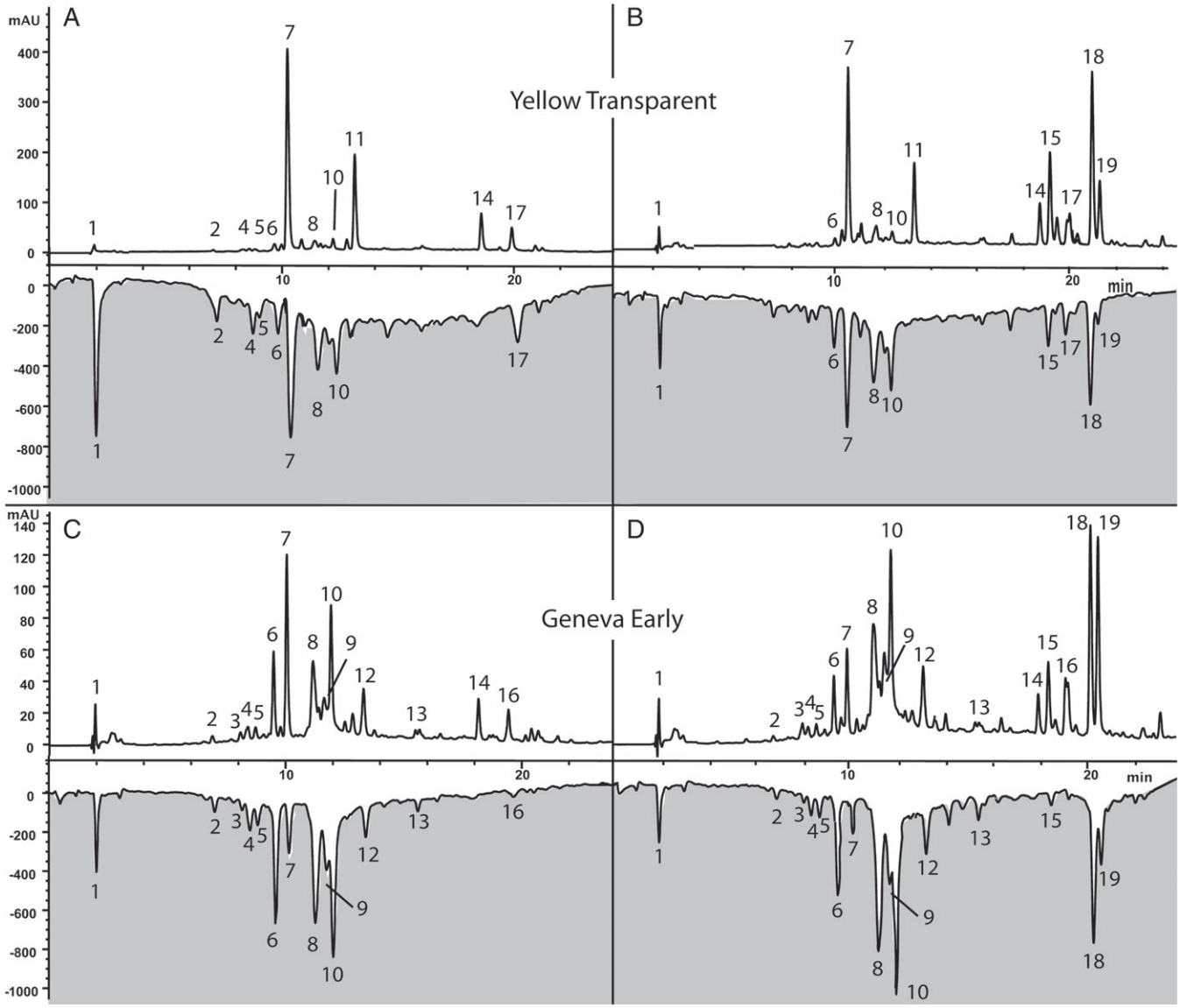

Figure 1. Sample HPLC-DAD chromatograms of flesh (A, C) and peel (B, D) of two apple cultivars protected by effective microorganisms (top chromatograms at 280 nm) along with profiles of antioxidants detected online with ABTS reagent (bottom chromatograms at 734 nm). For identity of peaks see Table 1.

Total antioxidant activity

The total antioxidant activities of apple samples derived from the two orchards, determined by three most popular spectrophotometric tests, are presented in Fig. 2. For all samples studied, the DPPH test gave the lowest values of Trolox equivalents (TE μmol g^{-1} DW), ranging from 11 to 34 μmol TE g^{-1} DW. The antioxidant activities measured by ABTS and F-C tests were similar and ranged from 16 to 83 μmol TE g^{-1} DW and from 27 to 104 μmol TE g^{-1} DW, respectively. The respective ranges calculated for fresh weight are as follows: 3–9, 4–22 and 6–46 TE μmol g^{-1} FW, for DPPH, ABTS and F-C, respectively. These results are in accordance with those by Vieira *et al.*[40] who reported the total antioxidant capacity measured with the ABTS assay for three different cultivars of apples to range from 5.37 in flesh to 20.99 μmol TE g^{-1} FW in peel samples. The total antioxidant activity of the peel was greater than that of the flesh for all pairs of apple samples studied, confirming the apparent induction of biosynthesis of antioxidant phytochemicals in response to microbial challenge.

The samples of apples protected with EM repeatedly showed higher antioxidant activity than corresponding samples protected with chemical pesticides (Fig. 2). The values determined with the ABTS test for samples from the EM-protected orchard in the case of 'Yellow Transparent' were for flesh and peel 70% and 62% higher than the same kind of samples from conventionally treated cultivar. For 'Geneva Early', these values were also higher by about 34% and 40%.

In general, the antioxidant potential of apple samples studied matched the content of polyphenols. Such results are not surprising, since it is well established that a strong positive relationship exists between the abundance of polyphenols in an apple sample and its antioxidant activity.[41,42] The same conclusion comes from our analyses, where a strong correlation was observed between the total antioxidant activity and the total content of polyphenols found in the studied apple samples, as shown by the Pearson coefficients: ABTS, $r = 0.91$; F-C, $r = 0.97$; DPPH, $r = 0.90$.

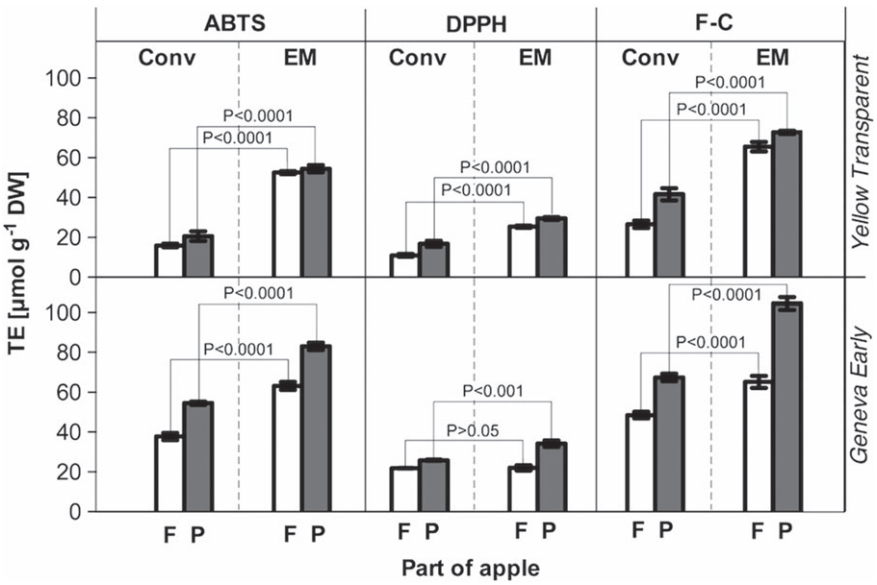

Figure 2. Total antioxidant activity of flesh (F) and peel (P) of two cultivars of apple (Yellow Transparent and Geneva Early) derived from orchards protected by conventional (Conv) pesticides or by effective microorganisms (EM) determined by ABTS, F-C and DPPH spectrophotometric tests. The results are expressed as Trolox equivalents (TE) and are the means ± SD of three independent determinations.

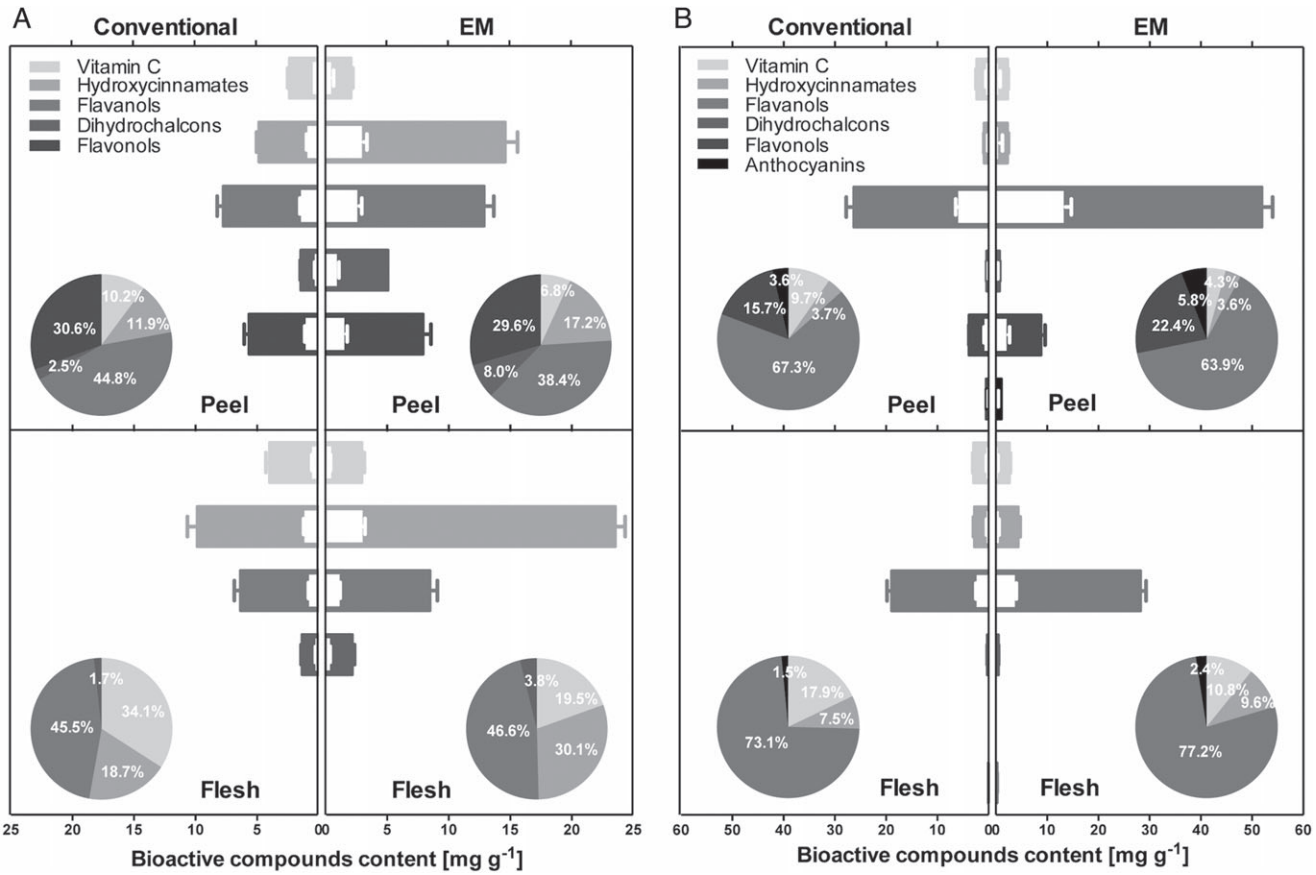

Figure 3. Comparison of the contents of major groups of bioactive compounds (calculated as mg g^{-1} of DW, grey bars, and mg g^{-1} FW, inner white bars) combined with the contribution of each group to the antioxidant activity (% of total) (pie graphs) in flesh and peel of Yellow Transparent (A) and Geneva Early (B) cultivars derived from orchards protected by conventional pesticides or by effective microorganisms (EM).

Table 2. Content of total lipids (mg g^{-1} DW) and classes of lipids (% of total lipids) in seeds and peels of two apple cultivars [Geneva Early (GE) and Yellow Transparent (YT)]

Sample	Content of lipids (mg g^{-1} DW)	Classes of lipids (% of total lipids)					
		Waxes	TAG	Sterol esters	MAG + DAG	Phospholipids	Phytosterols
Seeds							
Conv (YT)	74.8 ± 1.2	ND	97.66 ± 0.80	0.18 ± 0.11	ND	0.28 ± 0.12	0.38 ± 0.02
EM (YT)	75.7 ± 0.8	ND	97.03 ± 1.02	0.10 ± 0.04	0.66 ± 0.11	0.24 ± 0.06	0.49 ± 0.04
Significance	NS	–	NS	NS	NS	NS	NS
Conv (GE)	84.7 ± 0.9	ND	97.98 ± 1.47	0.02 ± 0.01	0.04 ± 0.02	0.10 ± 0.03	0.37 ± 0.02
EM (GE)	80.3 ± 1.1	ND	98.47 ± 1.29	0.17 ± 0.09	0.13 ± 0.04	0.11 ± 0.09	0.65 ± 0.02
Significance	***	–	NS	NS	NS	NS	NS
Peel							
Conv (YT)	82.2 ± 1.2	13.89 ± 0.39	79.96 ± 1.19	ND	0.02 ± 0.02	4.89 ± 1.20	0.02 ± 0.03
EM (YT)	69.2 ± 1.1	9.97 ± 0.23	82.95 ± 1.13	0.05 ± 0.02	0.11 ± 0.02	5.23 ± 1.23	0.42 ± 0.05
Significance	****	****	***	NS	NS	NS	NS
Conv (GE)	38.3 ± 1.0	13.70 ± 0.30	80.62 ± 0.95	0.02 ± 0.01	0.02 ± 0.03	4.11 ± 0.93	0.31 ± 0.03
EM (GE)	34.6 ± 0.9	13.92 ± 0.33	80.29 ± 0.79	0.05 ± 0.03	0.09 ± 0.03	4.18 ± 1.08	0.25 ± 0.02
Significance	**	NS	NS	NS	NS	NS	NS

Conv, conventional orchard protection; EM, microbiological orchard protection.
Significance, significant differences between corresponding samples (Conv vs. EM); $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; NS, not significant; ND, not detected.

Chromatographic profiles of antioxidants

Over the past two decades, a number of analytical methods measuring total antioxidative activity have been developed, one of them based on the ability of an antioxidant to quench ABTS radicals. The same chemical reaction has been exploited for online HPLC-coupled method that enables profiling of antioxidants in complex mixtures following their chromatographic separation from the matrix.[14–16] As in ABTS colorimetric assays, the reduction reaction leads to a significant shift in the UV–visible spectrum, so the change in absorption – discolouring of ABTS reagent – can serve as a quantitative measure of antioxidative potential of analytes separated by HPLC. This approach has been applied here for the detection of antioxidant phytochemicals in chromatographic profiles obtained for apple samples studied (Fig. 1, bottom chromatograms). The occurrence of antioxidants in eluate leads to negative peaks at 734 nm.

Chromatographic profiling coupled with chemical post-detection not only reveals the individual reducing analytes, but also enables quantification of their input into the antioxidant potential of the sample. In Fig. 3, the abundance of individual groups of polyphenols and their input into the antioxidant activity of peel and flesh samples of Yellow Transparent and Geneva Early cultivars are jointly presented. Histograms illustrate the contents of major groups of bioactive components in DW (grey bars) and their abundance calculated for FW (inner white bars). In pie graphs, the percentage contribution of the particular group of compounds to the antioxidant activity of apple samples is presented. Generally, this comparison confirms the increase in the abundance of polyphenols in fruits that were in contact with greater amount of microorganisms. The data assembled in Fig. 3 also suggest that apples should be consumed with peel, as it is a major source of health beneficial phytochemicals in these fruits.

The main contributors to the total antioxidant activity of both flesh and peel samples are flavanols (38–47% in 'Yellow Transparent' and 64–77% in 'Geneva Early'). Hydroxycinnamate inputs ranged from 12% to 30% in Yellow Transparent cultivar and from 4% to 10% in Geneva Early. In the case of peel samples, of both cultivars, flavonols provided 16–31% of contribution to the antioxidant activity. The lowest impact on antioxidant activity had dihydrochalcons in 'Yellow Transparent' (2–8%) and anthocyanins in 'Geneva Early' (2–6%). These results are similar to the levels reported by Tsao *et al.*[43] who calculated the contribution of phenolic fraction to the antioxidant activity of apple as follows: in flesh, 69% flavan-3-ols, 31% hydroxycinnamic acids, <0.1% quercetin glycosides and dihydrochalcones; in peels: 71% flavan-3-ols, 12% quercetin glycosides, 10% cyanidin-3-galactoside, 7% hydroxycinnamic acids and 0.1% dihydrochalcones.

Though dihydrochalcones are not the major polyphenols determined, interestingly their input into the total antioxidant activity is consistently greater in apple samples derived from EM-protected compared to pesticide protected orchard. In contrast, the use of microorganisms for the orchard protection results in the decrease of both vitamin C content (Table 1) and its contribution to the total antioxidant activity (Fig. 3).

Content and classes of lipids

Another group of metabolites assessed were lipids present in seeds and peel of apples studied. In contrast to polyphenols, lipids are primary metabolites, so they are generated constitutively. Therefore, as mentioned previously, their content was regarded as a control of natural variability of the phytomes of studied apples. The total content of lipids in the seeds of two varieties of apples derived from the two orchards (protected conventionally or by EM) ranged from 74.8 to 84.7 mg g^{-1} DW (Table 2). As expected, seed lipids did not seem to be affected by the method of orchard protection. Their abundance was rather characteristic for the cultivar and constituted for 'Geneva Early' and 'Yellow Transparent' about 82.0 and 75.0 mg g^{-1} DW, respectively.

The greater diversification of the total contents of lipids was observed in the peels, where it reached statistical significance in the case of total lipids. The peels of Geneva Early cultivar

Table 3. Fatty acids composition in seeds and peels of two apple cultivars [Geneva Early (GE) and Yellow Transparent (YT)] derived from orchards protected by conventional pesticides (Conv) or by effective microorganisms (EM) (% of total fatty acids)

	Seeds						Peel					
	YT			GE			YT			GE		
Fatty acid	Conv	EM	*P*	Conv	EM	*P*	Conv	EM	*P*	Conv	EM	*P*
12:0	0	0	NS	0	0	NS	0.85	0.32	NS	0.70	0.76	NS
14:0	0.05	0	NS	0.02	0.05	NS	0.56	0.76	NS	1.02	1.06	NS
16:0	7.73	7.31	NS	8.02	7.15	NS	13.54	13.30	NS	13.81	12.68	NS
16:1 9c	0	0.05	NS	0.05	0.06	NS	0	0	NS	0	0	NS
18:0	1.59	1.47	NS	1.75	1.96	NS	12.53	16.25	****	9.11	8.79	NS
18:1 9c	35.29	37.47	*	37.94	38.56	NS	17.92	12.24	****	15.63	13.26	**
18:2 (*n*-6)	51.40	50.71	NS	49.22	48.91	NS	37.85	36.86	NS	40.83	45.07	****
18:3 (*n*-3)	0.51	0.42	NS	0.31	0.42	NS	5.08	6.04	NS	5.45	6.75	NS
20:0	1.49	1.17	NS	1.54	1.51	NS	8.55	11.7	****	8.28	7.05	NS
20:1 9c	0.56	0.53	NS	0.48	0.55	NS	0.37	0.22	NS	1.41	0.93	NS
20:2	0.06	0.08	NS	0.04	0.1	NS	0.05	0.03	NS	0.59	0.32	NS
22:0	0.29	0.21	NS	0.27	0.40	NS	1.46	1.84	NS	2.62	2.61	NS
24:0	0.04	0.04	NS	0.03	0.05	NS	0	0	NS	0	0	NS
Sum of FA	99.01	99.46	NS	99.77	99.62	NS	98.76	98.83	NS	99.45	99.28	NS
SFA	11.19	10.20	NS	11.63	11.12	NS	37.49	43.54	NS	35.54	32.95	NS
MUFA	35.85	38.05	NS	38.47	39.17	NS	18.29	12.46	NS	17.04	14.9	NS
PUFA	51.97	51.21	NS	49.67	49.33	NS	42.98	42.83	NS	46.87	52.14	NS

P, significant differences between corresponding samples (Conv vs. EM); *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$; NS, not significant.
SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
The results are means (% of total fatty acids) calculated from three independent experiments.

contained on average 36.0 mg g^{-1} DW lipids regardless of the crop protection. In contrast, the total content of lipids in the case of peels of Yellow Transparent cultivar derived from the EM-protected orchard was lower by 15% from that determined for apples derived from the conventionally protected orchard. Considering other results, the peel from the latter sample contained close to 4% less waxes than corresponding conventionally protected apple sample (contents of waxes about 13.8% of total lipids in Conv. vs. 9.9% in EM 'Yellow Transparent'). The plant cuticle forms a protective layer that covers primary aerial surfaces of all land plants and protects plant against water loss, weathering, abrasions, as well as against infection, plant pathogens or insect herbivores.[44] Protective layers containing the waxes can easily be removed from fruit cuticle, usually unintentionally, e.g. during collection of fruits or washing. The high decrease in the content of waxes observed for 'Yellow Transparent' apples protected with EM suggests such an unintentional removal of this class of lipids from apple cuticle, prior to the preparation of the sample, which could impact the calculated statistical significance.

The results obtained do not indicate that a method of protection of orchards during fruit ripening had any significant effect on the composition of lipid classes in seeds. The most noticeable difference was the lack of waxes in seeds; however, also the content of lipids representing particular classes varied (Table 2). The main lipid fraction in both analysed parts of apples was TAGs (on average 97% in the oil from seeds and about 80% in the lipids from peels). When it comes to bioactive lipids of which apples are a known valuable source,[45] phospholipids were more abundant in peels (4.11–5.23%), while phytosterols in seed oil (0.37–0.65%). In both cases, the samples derived from EM-protected fruits tended to contain slightly more of these bioactive components.

Fatty acids composition

The typical composition of fatty acids in apple seed oils reveals the dominance of linoleic acid 18:2 (range from 43% to 55% of total fatty acids), oleic acid C18:1 (from 25% to 37%) and palmitic acid C16:0 (5.5–8%).[46,47] Similar shares were determined for the apple samples studied here (Table 3). The content of linoleic acid in oil from 'Yellow Transparent' apple seeds, no matter the orchard protection, amounted to approx. 51% and in 'Geneva Early' apple seeds approx. to 49%. A somewhat lower level of this acid was found in the lipids obtained from the peels of the 'Yellow Transparent' apples, approx. 37% in the case of both orchards. The peels of the 'Geneva Early' apples contained 40.8% and 45.0% of linoleic acid in the lipids obtained from fruits protected using pesticides and by EM, respectively.

Total lipids determined for the peels of apples showed more than 6% higher content of palmitic acid compared to the seed oils, and over 10% higher content of stearic acid. As a result, the main fatty acids of apple seed oils studied were PUFA (approx. 50%), while in the case of peels of apples, equally abundant were saturated fatty acids (approx. 30%). No significant impact of the method of protection on fatty acid composition in apple samples studied was seen.

CONCLUSIONS

This study shows that effective microorganisms applied for the orchard protection can increase the content of polyphenols, as well as antioxidant activity of apples. These results can be explained by the impact of microorganisms or their metabolites or both on regulation of gene pathways involved in biosynthesis of secondary metabolites (e.g. phenylpropanoid pathway and flavonoid pathway, a branch of the phenylpropanoid pathway).[12]

The results indicate that the advantage of using the EM technology in agriculture may not only be the reduction of consumption of chemical fertilisers and synthetic pesticides, but also, at least in the case of apples, may lead to production of crops with improved health quality due to the higher content of bioactive phytochemicals.

It may be worth to note that fresh or cold-pressed produce (e.g. juices) and wastes of EM-protected apples may be also enriched in prebiotic microorganisms contained in EM-preparation. Health benefits of phytoantioxidants combined with EM in a form of fermented drink sold in East Asia (beverage EX-X and EM-YU) have been well documented *in vitro* and *in vivo*. These drinks exhibited the ability to inhibit inflammation,[48] to prevent asthma,[48] osteoporosis,[49] Parkinson's disease[50] and cancer cell growth[51] without showing any toxicity as demonstrated in appropriate animal models.[52] These drinks also displayed antibacterial activity including reduction of the biofilm formation.[53] Our results indicate that, additionlly, apples and their leftovers may serve as raw material for production of similar health promoting foods.

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